

REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: 3AEY

Product name(s): Mevalone

Chemical active substances:

Eugenol 33 g/L

Geraniol 66 g/L

Thymol 66 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(Authorization for Mevalone product)

Applicant: Eden Research plc

Submission date: 15/07/2021

Update date: 21/12/2021

MS Finalisation date: April 2022 (initial Core Assessment)

November 2022 (final Core Assessment)

Version history

When	What
July 2021	Authorization of marketing in Central Zone of the plant protection product Mevalone on grapes and pome fruits
December 2021	Addition of storage stability final reports for grapes and apples. Addition of validation methods in apples, body fluids and body tissues. Remove of the validation methods in soil.
April 2022	Initial zRMS assessment The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are struck through and shaded for transparency .
November 2022	Final report (Core Assessment updated following the commenting period). No additional information or assessments after the commenting period.

Table of Contents

5	Analytical methods.....	5
5.1	Conclusion and summary of assessment	5
5.2	Methods used for the generation of pre-authorization data (KCP 5.1).....	7
5.2.1	Analysis of the plant protection product (KCP 5.1.1)	7
5.2.1.1	Determination of active substance and/or variant in the plant protection product (KCP 5.1.1).....	7
5.2.1.2	Description of analytical methods for the determination of relevant impurities (KCP 5.1.1).....	29
5.2.1.3	Description of analytical methods for the determination of formulants (KCP 5.1.1).....	38
5.2.1.4	Applicability of existing CIPAC methods (KCP 5.1.1)	38
5.2.2	Methods for the determination of residues (KCP 5.1.2).....	38
5.3	Methods for post-authorization control and monitoring purposes (KCP 5.2)	44
5.3.1	Analysis of the plant protection product (KCP 5.2)	44
5.3.2	Description of analytical methods for the determination of residues of eugenol (KCP 5.2).....	45
5.3.2.1	Overview of residue definitions and levels for which compliance is required.....	45
5.3.2.2	Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)	45
5.3.2.3	Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)	46
5.3.2.4	Description of methods for the analysis of soil (KCP 5.2).....	47
5.3.2.5	Description of methods for the analysis of water (KCP 5.2).....	47
5.3.2.6	Description of methods for the analysis of air (KCP 5.2)	47
5.3.2.7	Description of methods for the analysis of body fluids and tissues (KCP 5.2)	48
5.3.2.8	Other studies/ information	48
5.3.3	Description of analytical methods for the determination of residues of geraniol (KCP 5.2).....	48
5.3.3.1	Overview of residue definitions and levels for which compliance is required.....	48
5.3.3.2	Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)	50
5.3.3.3	Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)	50
5.3.3.4	Description of methods for the analysis of soil (KCP 5.2).....	51
5.3.3.5	Description of methods for the analysis of water (KCP 5.2).....	51
5.3.3.6	Description of methods for the analysis of air (KCP 5.2)	51
5.3.3.7	Description of methods for the analysis of body fluids and tissues (KCP 5.2)	52
5.3.3.8	Other studies/ information	52
5.3.4	Description of analytical methods for the determination of residues of thymol (KCP 5.2).....	52
5.3.4.1	Overview of residue definitions and levels for which compliance is required.....	52
5.3.4.2	Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)	53
5.3.4.3	Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)	54
5.3.4.4	Description of methods for the analysis of soil (KCP 5.2).....	55
5.3.4.5	Description of methods for the analysis of water (KCP 5.2).....	55
5.3.4.6	Description of methods for the analysis of air (KCP 5.2)	55
5.3.4.7	Description of methods for the analysis of body fluids and tissues (KCP 5.2)	55
5.3.4.8	Other studies/ information	56
Appendix 1	Lists of data considered in support of the evaluation.....	57
Appendix 2	Detailed evaluation of submitted analytical methods.....	67

A 2.1	Analytical methods for eugenol.....	67
A 2.1.1	Methods used for the generation of pre-authorization data (KCP 5.1).....	67
A 2.1.2	Methods for post-authorization control and monitoring purposes (KCP 5.2)	128
A 2.2	Analytical methods for geraniol	201
A 2.2.1	Methods used for the generation of pre-authorization data (KCP 5.1).....	201
A 2.2.2	Methods for post-authorization control and monitoring purposes (KCP 5.2)	264
A 2.3	Analytical methods for thymol	324
A 2.3.1	Methods used for the generation of pre-authorization data (KCP 5.1).....	324
A 2.3.2	Methods for post-authorization control and monitoring purposes (KCP 5.2)	391

5 Analytical methods

5.1 Conclusion and summary of assessment

zRMS conclusion:

Eugenol

According to the EFSA Journal 2012;10(11):2914: “Due to the nature of the compound no methods are required for products of plant and animal origin. However, for soil and water methods are required (see section 4). For air a method of analysis is required as there is an exposure scenario. Methods of analysis for body fluids and tissues are not required as the active substance is not considered as toxic or very toxic.”

Analytical methods for residues (Regulation (Annex IIA, point 4.2)

Residue definitions for monitoring purposes

Food of plant origin	Currently not required (see section 3)
Food of animal origin	Not required
Soil	Eugenol, open for other compounds.
Water surface	Eugenol, open for other compounds.
drinking/ground	Eugenol, open for other compounds.
Air	Eugenol, open for other compounds.

Monitoring/Enforcement methods

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	Not required
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	Not required
Soil (analytical technique and LOQ)	Open
Water (analytical technique and LOQ)	Open
Air (analytical technique and LOQ)	Open
Body fluids and tissues (analytical technique and LOQ)	Not required as the active substance is not classified as toxic or very toxic.

Geraniol

According to the EFSA Journal 2012;10(11):2915 “Due to the nature of the compound no methods are required for products of plant and animal origin. However, methods are required for soil and water (see section 4). For air, as there is an exposure scenario, a method of analysis is required. Methods of analysis for body fluids and tissues are not required as the active substance is not considered as toxic or very toxic.”

Analytical methods for residues (Regulation (Annex IIA, point 4.2)

Residue definitions for monitoring purposes

Food of plant origin	Currently not required (see section 3)
Food of animal origin	Not required
Soil	Geraniol
Water surface	Geraniol
drinking/ground	Geraniol
Air	Geraniol

Monitoring/Enforcement methods

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	Not required as no residue definition currently proposed.
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	Not required
Soil (analytical technique and LOQ)	Open
Water (analytical technique and LOQ)	Open
Air (analytical technique and LOQ)	Open
Body fluids and tissues (analytical technique and LOQ)	No methods proposed or required as geraniol is not classified as toxic (T) or very toxic (T+).

Thymol

According to the EFSA Journal 2012;10(11):2916: “Due to the nature of the compound no methods are required for products of plant and animal origin. However, for soil and water methods are required (see section 4). A method of analysis is required for air as there is an exposure scenario. Methods of analysis for body fluids and tissues are not required as the active substance is not considered as toxic or very toxic.”

Analytical methods for residues (Regulation (Annex IIA, point 4.2)

Residue definitions for monitoring purposes

Food of plant origin	Currently not required (see section 3)
Food of animal origin	Not required
Soil	Thymol
Water surface	Thymol
drinking/ground	Thymol
Air	Thymol

Monitoring/Enforcement methods

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	Methods not required as no residue definition currently proposed.
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	Not required as no residue definition proposed.
Soil (analytical technique and LOQ)	Open
Water (analytical technique and LOQ)	Open
Air (analytical technique and LOQ)	Open
Body fluids and tissues (analytical technique and LOQ)	Not required as the active substance is not classified as toxic or very toxic.

Conclusion:

In the EU review it was concluded that enforcement methods are not required for eugenol, geraniol and thymol residues in products of plant and animal origin.

Geraniol, thymol and eugenol are naturally occurring substances in plants. In view of this, it was considered appropriate to include this substance temporarily in Annex IV to Regulation (EC) No 396/2005 pending submission of the reasoned opinion of the Authority in accordance with Article 12.

According to the EFSA Journal 2012;10(11):2914-2916 the data gap is identified for methods of analysis for soil, water and air for eugenol, geraniol and thymol.

New analytical methods for the determination of

- eugenol and methyl-eugenol residues in plant matrices,
- eugenol and methyl-eugenol residues in animal matrices,
- eugenol and methyl-eugenol residues in water,
- eugenol and methyl-eugenol residues in air,
- eugenol and methyl-eugenol residues in body fluids and tissues,
- geraniol residues in plant matrices,
- geraniol residues in animal matrices,
- geraniol residues in water,
- geraniol residues in air,
- geraniol residues in body fluids and tissues,
- thymol residues in plant matrices,
- thymol residues in animal matrices,
- thymol residues in water,
- thymol residues in air,
- thymol residues in body fluids and tissues

have been provided by Applicant. These data are currently under evaluation for the renewal of approval of the active substances. For the detailed evaluation of new studies it is referred to Appendix 2. The studies are acceptable.

According to the SANCO/825/00 rev.8.1 and the guidance document SANTE/2020/12830 rev.1 “A validation of the primary monitoring method in an independent laboratory (ILV) is required for the determination of residues in food of plant and animal origin and in drinking water. The ILV shall confirm the LOQ of the primary method, or at least cover the lowest MRL.” It should be noted that no definition of residue or MRL has been set for the active substance eugenol, geraniol and thymol in food and feed of animal origin, so the analytical methods and ILV for

plant and animal matrices are not required.

Study of ILV for the determination of eugenol, methyl-eugenol, geraniol and thymol residues in water is on-going and will be submitted as part of the renewal of approval of active substances.

Sufficiently sensitive and selective analytical methods are available for the active substances and relevant impurities in the plant protection product.

Sufficiently sensitive and selective analytical methods are not required for all analytes included in the residue definitions as no definition of residue or MRL has been set for the active substances eugenol, geraniol and thymol.

Commodity/crop	Supported/ Not supported
Grapes	Supported (no residue definition or MRL)
Pome fruits	Supported (no residue definition or MRL)

Noticed data gaps are:

- ILV for the determination of eugenol, methyl-eugenol, geraniol and thymol residues in water is required and should be provided at the renewal of active substances.

5.2 Methods used for the generation of pre-authorization data (KCP 5.1)

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

5.2.1.1 Determination of active substance and/or variant in the plant protection product (KCP 5.1.1)

The following analytical methods for the determination of eugenol, geraniol and thymol in the plant protection product Mevalone have previously been reviewed (within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU) and are provided in support of this assessment:

- analytical method M619 for eugenol, geraniol and thymol determination in Mevalone
- analytical method TS20010-1 for eugenol, geraniol and thymol determination in Mevalone
- analytical method for active substances release from encapsulated formulation Mevalone

• Method M619

Comments of zRMS:	Analytical method and its validation has already been evaluated during the EU approval process of the active substance. The analytical method M619 was successfully validated for the determination of eugenol, geraniol and thymol in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4. Validation complies with SANCO/3030/99 rev.5.
-------------------	--

Data point:	CP 5.1.1/01 (5.2.1.1/01 of this dRR)
Report author	White, G.A.
Report year	2007d
Report title	Validation of Analytical Method M619, Gas chromatographic determination of free and encapsulated thymol, eugenol and geraniol in formulations, for the 3AEY formulation
Report No	J16312
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4

Deviations from current test guideline None

Previous evaluation Yes, evaluated and accepted in DAR (2011) under data point IIIA 5.2.1/01

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

Eugenol, geraniol and thymol are determined and quantified using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard. The method allows for separate quantification of free and encapsulated eugenol, geraniol and thymol.

Test material

Test material

Test item

Name: 3AEY
Source and lot/batch no.: Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content: Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg)
Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg)
Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)
Expiry date of lot/batch: December 2008
Storage conditions: Ambient temperature

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: Eden research plc, lot n°95217, purity 98.8%
Expiry date: not stated
Storage conditions: In a refrigerated dessicator

Name: Geraniol technical
CAS number: 89-83-8
Source and lot/batch no.: Eden research plc, lot n°96904, purity 98.2%
Expiry date: April 2008
Storage conditions: In a refrigerated dessicator

Name: Thymol technical
CAS number: 89-83-8
Source and lot/batch no.: Eden research plc, lot n°94747, purity 99.7%
Expiry date: not stated
Storage conditions: In a refrigerated dessicator

Reference material

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: Riedel-de Haën., lot n°3071X, purity 99.5%
Expiry date: March 2010
Storage conditions: deep freeze conditions

Name: Geraniol
CAS number: 89-83-8
Source and lot/batch no.: Fluka, lot n°1151550, purity 99.0%
Expiry date: February 2008
Storage conditions: In a refrigerated dessicator

Name: Thymol
CAS number: 89-83-8

Source and lot/batch no.: Fluka, lot n°1130656, purity 99.9%
Expiry date: April 2008
Storage conditions: deep freeze conditions

Analysis parameters

Method type: GC-FID
Instrument: GC, 5890 (Hewlett Packard) with flame ionisation detector (FID)
Analytical column: Zebron ZB-5, 30 m x 0.32 mm, 1.0 µm film thickness or equivalent
Oven: 50°C -5°C/min – 180°C
Detector temperature: 280°C
Injector temperature: 160°C
Injection volume: 2 µL
Injection mode: Split 20:1 using SGE Focusliner
Carrier gas: Oxygen-free nitrogen
Flow rate: 1.8 mL/min
Retention time: 1-nonanol (Internal Standard): Approx. 18 min
Eugenol: Approx. 22 min
Geraniol: Approx. 21 min
Thymol: Approx. 22 min

Sample preparation

Total active substances content:

Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID.

Free active substances content:

A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000 g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID.

Stock solutions and calibration standards

- Internal standard solutions:

Total active substances content internal standard solution: 2.5 g of 1-nonanol are transferred to a 1L volumetric flask, dissolved in and adjusted to volume with methanol. This solution is referred to as IS1.

Free active substances content internal standard solution: 0.5 g of 1-nonanol is transferred into a 200 mL volumetric flask, dissolved in and adjusted to volume with hexane. This internal standard is used directly for the preparation of calibration standards. This solution is referred to as IS2.

Free active substances content internal standard solution for sample analysis: pipette 25 mL of IS2 into a 500 mL volumetric flask and make up to volume with hexane. This solution is referred to as IS3.

- Eugenol standard solutions:

Total eugenol Standard Solutions: for an expected total eugenol content of 3.3% w/w in the test item, analytical grade eugenol (0.01 to 0.04 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS1. The calibration range spans concentrations from 0.5 mg/mL to 2 mg/mL of eugenol. Seven calibration standards are prepared.

Free eugenol Standard Solutions: for an expected free eugenol content of 0.09% in the test item, analytical grade eugenol (0.01 to 0.04 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS2. 1 mL of each solution is diluted into 20 mL hexane, providing a calibration range over the 0.025 mg/mL to 0.1 mg/mL bracket. Six calibration standards are prepared.

- **Geraniol standard solutions:**

Total geraniol standard solutions: for an expected total geraniol content of 6.6% in the test item, analytical grade geraniol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS1. The calibration range spans concentrations from 1 mg/mL to 4 mg/mL. Seven calibration standards are prepared.

Free geraniol standard solutions: for an expected free geraniol content of 0.13% in the test item, analytical grade geraniol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS2. 1 mL of each solution is diluted into 20 mL hexane, providing a calibration range over the 0.05 mg/mL to 0.2 mg/mL bracket. Six calibration standards are prepared.

- **Thymol standard solutions:**

Total thymol Standard Solutions: for an expected total thymol content of 6.6% in the test item, analytical grade thymol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS1. The calibration range spans concentrations from 1 mg/mL to 4 mg/mL of thymol. Seven calibration standards are prepared.

Free thymol Standard Solutions: for an expected free thymol content of 0.13% in the test item, analytical grade thymol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS2. 1 mL of each solution is diluted into 20 mL hexane, providing a calibration range over the 0.05 mg/mL to 0.2 mg/mL bracket. Six calibration standards are prepared.

Calculations

The same calculations are applied to calculate total active substances and free active substances content. Concentration of active substances is calculated as follows:

$$\text{A. S. content} = \frac{\text{AE}}{\text{AIS}} \times \frac{\text{Wt IS} \times 100}{\text{Wts} \times \text{FAv.}}$$

Where:

- A.S. content = active substance content in % w/w
- AE = area active substance peak in sample
- AIS = area Internal Standard peak in sample
- Wt IS = Weight of Internal Standard solution dilution
- Wts = Weight of sample taken
- FAv. = average Response Factor from the internal standards before and after the sample

To calculate the amount of encapsulated active substances, the amount of free active substances is subtracted from the amount of total active substances.

Accuracy and repeatability

Accuracy was assessed through six independent samples. Technical active substances are accurately weighed into a 100 mL volumetric flask, in quantities of between $\pm 25\%$ of the nominal active substances content expected to be found in the formulation. Test item blank formulation is accurately weighed into each flask at a level similar to that used for sample analysis (i.e. between 0.7 and 0.8 g). The contents of the flasks were homogenized carefully and the whole sample is subjected to sample preparation as described above.

The same technical active substances are used to prepare the calibration standards used for accuracy and repeatability determination.

Repeatability (precision):

For free active substances analysis precision, the formulation was centrifuged as described in the sample preparation section; seven replicate samples of the upper layer covering the range of about $\pm 50\%$ of the nominal free active substances content were weighed out, extracted with hexane and quantified as described above.

For total active substances analysis precision, six replicate samples covering the range of about $\pm 25\%$ of the nominal total active substances content were weighed out and prepared as described above.

Confirmation of substance identification

Confirmation of substance identification was performed by GC-MS under conditions as close to the

original quantification conditions as possible. Eugenol, geraniol and thymol were successfully identified against analytical standards.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances eugenol, geraniol and thymol in plant protection product Mevalone

	Eugenol	Geraniol	Thymol
Author(s), year	White, G.A., 2007		
Principle of method	Gas chromatography with a flame-ionisation detector (GC-FID) and 1-nonanol as internal standard		
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	0.025 – 0.10 mg/mL: $y = 18.010 x + 0.0001$ 0.5 - 2.0 mg/mL: $y = 17.519 x - 0.0019$ $y = \text{peak area ratio (eugenol area/1-nonanol area), } x = \text{weight of eugenol (in g)}$ $R \geq 0.9998$	1.0 – 4.0 mg/mL: $y = 20.957x + 0.0002$ $y = \text{peak area ratio (geraniol area/1-nonanol area), } x = \text{weight of geraniol (in g)}$ $R = 1.000$	0.05 – 0.20 mg/mL: $y = 21.455 x + 0.0077$ 1.0 - 4.0 mg/mL: $y = 21.069 x + 0.0096$ $y = \text{peak area ratio (thymol area/1-nonanol area), } x = \text{weight of thymol (in g)}$ $R \geq 0.9999$
Precision – Repeatability Mean (%RSD)	Total eugenol (n = 7): 3.585% w/w (RSD: 0.522%) Horrat = 0.24 Free eugenol (n = 6): 0.098% w/w (RSD: 0.684%) Horrat = 0.18	Total geraniol (n = 7): 6.610% w/w (RSD: 0.395%) Horrat = 0.19 Free geraniol (n = 6): 0.125% w/w (RSD: 0.781%) Horrat = 0.21	Total thymol (n = 7): 6.695% w/w (RSD: 0.441%) Horrat = 0.22 Free geraniol (n = 6): 0.138% w/w (RSD: 1.407%) Horrat = 0.39
Accuracy (% Recovery)	100.37% (n = 6) (RSD = 2.24%) Horrat = 0.30	99.33% (n = 6) (RSD = 0.702%) Horrat = 0.34	100.25% (n = 6) (RSD = 0.901%) Horrat = 0.44
Interference/ Specificity	No interferences were observed at the retention time of eugenol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.	No interferences were observed at the retention time of geraniol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.	No interferences were observed at the retention time of thymol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.
Confirmation	The retention times and mass spectra for the peaks eluting at the retention times ascribed to eugenol were compared for both the standard and test item sample injections.	The retention times and mass spectra for the peaks eluting at the retention times ascribed to geraniol were compared for both the standard and test item sample injections.	The retention times and mass spectra for the peaks eluting at the retention times ascribed to thymol were compared for both the standard and test item sample injections.

Conclusion

The validation of the method for analysis of eugenol, geraniol and thymol in 3AEY has already been evaluated at EU level. It was performed under GLP according to Guideline SANCO/3030/99 rev.4 and was successfully validated. Validation also complies with SANCO/3030/99 rev.5.

The method is acceptable for the quantification of eugenol, geraniol and thymol in 3AEY.

Comments of zRMS:	Analytical method and its validation has already been evaluated during the EU approval process of the active substance. The proposed analytical method was successfully validated for the determination of eugenol, geraniol and thymol (free and total active
-------------------	--

	substances) in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4.
--	--

Data point:	CP 5.1.2/15 (5.2.1.1/02 of this dRR)
Report author	White, G.A.
Report year	2011
Report title	3AEY Formulation Storage Stability Trial and Physical / Chemical Tests Final Report (24 Months Storage)
Report No	J16313
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 2.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol, geraniol and thymol are determined and quantified using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard. The method used is referenced as M619. The method allows for separate quantification of free and encapsulated eugenol. The method is fully summarised within White 2007d (report n°J16312), included in this submission.

Test material

Test material

Test Item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, batch n°YPOA3Y424-1.1 Geraniol (CAS no. 106-24-1): 6.61% w/w (66.1 g/kg) Thymol (CAS no. 89-83-8): 6.69% w/w (66.9 g/kg) Eugenol (CAS no. 97-53-0): 3.59% w/w (35.9 g/kg)
Expiry date of lot/batch:	not stated
Storage conditions:	not stated

Reference material

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Penta Manufacturing Co., lot n°95217, purity 98.8%
Expiry date:	August 2011
Storage conditions:	In a refrigerated dessicator

Name:	Geraniol
CAS number:	106-24-1
Source and lot/batch no.:	Penta Manufacturing Co., lot n°96904, purity 98.2%
Expiry date:	August 2011
Storage conditions:	In a refrigerated dessicator

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Penta Manufacturing Co., lot n°94747, purity 99.7%
Expiry date:	August 2011
Storage conditions:	In a refrigerated dessicator

Analysis parameters

Method type	GC-FID
Instrument:	GC, 5890 (Hewlett Packard) with flame ionisation detector (FID)
Analytical column:	Zebron ZB-5, 30 m x 0.32 mm, 1.0 µm film thickness or equivalent
Oven:	50°C (2 min) - 5°C/min – 180°C
Detector temperature:	280°C
Injector temperature:	160°C
Injection volume:	2 µL
Injection mode:	Split 10:1 using SGE Focusliner
Carrier gas:	Oxygen-free nitrogen
Flow rate:	3.5 mL/min
Retention time:	1-nonanol (Internal Standard): Approx. 18 min Eugenol: Approx. 22 min Geraniol: Approx. 21 min Thymol: Approx. 22 min

Sample preparation

- Eugenol content:

Total eugenol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal eugenol content of 3.3% w/w, the sample concentrations are 1.16 and 1.32 mg/mL respectively.

Free eugenol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free eugenol content of 0.09% w/w, extract concentrations are 0.063 and 0.090 mg/mL respectively.

- Geraniol standard content:

Total geraniol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal geraniol content of 6.6% w/w, the sample concentrations are 2.31 and 2.64 mg/mL respectively.

Free geraniol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free geraniol content of 0.13% w/w, extract concentrations are 0.091 and 0.13 mg/mL respectively.

- Thymol content:

Total thymol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal thymol content of 6.6% w/w, the sample concentrations are 2.31 and 2.64 mg/mL respectively.

Free thymol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free thymol content of 0.13% w/w, extract concentrations are 0.091 and 0.13 mg/mL respectively.

Method validation

Full description of the method's validation is provided within White 2007d (report n° J16312), which is included in this submission.

Conclusions

Total eugenol, geraniol and thymol are determined and quantified in 3AEY after dilution in methanol using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

Free eugenol, geraniol and thymol are determined and quantified in 3AEY after extraction in hexane using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

The method for quantification of eugenol in formulation was fully validated for specificity, linearity, accuracy and precision as part of study n°J16312 (White 2007d). The method is acceptable for the quantification of eugenol, geraniol and thymol (free and total active substances) in formulation 3AEY.

Comments of zRMS:	Analytical method and its validation has already been evaluated during the EU approval process of the active substance. The proposed analytical method was successfully validated for the quantification of eugenol, geraniol and thymol (free and total active substances) in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4.
-------------------	--

Data point:	CP 5.1.2/16 (5.2.1.1/03 of this dRR)
Report author	White, G.A.
Report year	2007c
Report title	3AEY Formulation accelerated and cold temperature storage stability trials and physical chemical tests
Report No	J16537
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 2.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol, geraniol and thymol are determined and quantified using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard. The method used is referenced as M619. The method allows for separate quantification of free and encapsulated eugenol. The method is fully summarised within White 2007d (report n°J16312), included in this submission.

Test material

Test material

Test Item

Name: 3AEY
Source and lot/batch no.: Eden Research plc, batch n°YPOA3Y424-1.1
Geraniol (CAS no. 106-24-1): 6.61% w/w (66.1 g/kg)
Thymol (CAS no. 89-83-8): 6.69% w/w (66.9 g/kg)

Eugenol (CAS no. 97-53-0): 3.59% w/w (35.9 g/kg)
Expiry date of lot/batch: not stated
Storage conditions: not stated

Reference material

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: Penta Manufacturing Co., lot n°95217, purity 98.8%
Expiry date: March 2008
Storage conditions: In a refrigerated dessicator

Name: Geraniol
CAS number: 106-24-1
Source and lot/batch no.: Penta Manufacturing Co., lot n°96904, purity 98.2%
Expiry date: March 2008
Storage conditions: In a refrigerated dessicator

Name: Thymol
CAS number: 89-83-8
Source and lot/batch no.: Penta Manufacturing Co., lot n°94747, purity 99.7%
Expiry date: March 2008
Storage conditions: In a refrigerated dessicator

Analysis parameters

Method type: GC-FID
Instrument: GC, 5890 (Hewlett Packard) with flame ionisation detector (FID)
Analytical column: Zebron ZB-5, 30 m x 0.32 mm, 1.0 µm film thickness or equivalent
Oven: 50°C (2 min) - 5°C/min – 180°C
Detector temperature: 280°C
Injector temperature: 160°C
Injection volume: 2 µL
Injection mode: Split 10:1 using SGE Focusliner
Carrier gas: Oxygen-free nitrogen
Flow rate: 3.5 mL/min
Retention time: 1-nonanol (Internal Standard): Approx. 18 min
Eugenol: Approx. 22 min
Geraniol: Approx. 21 min
Thymol: Approx. 22 min

Sample preparation

- Eugenol content:

Total eugenol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal eugenol content of 3.3% w/w, the sample concentrations are 1.16 and 1.32 mg/mL respectively.

Free eugenol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free eugenol content of 0.09% w/w, extract concentrations are 0.063 and

0.090 mg/mL respectively.

- **Geraniol standard content:**

Total geraniol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal geraniol content of 6.6% w/w, the sample concentrations are 2.31 and 2.64 mg/mL respectively.

Free geraniol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free geraniol content of 0.13% w/w, extract concentrations are 0.091 and 0.13 mg/mL respectively

- **Thymol content:**

Total thymol content: Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal thymol content of 6.6% w/w, the sample concentrations are 2.31 and 2.64 mg/mL respectively.

Free thymol content: A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free thymol content of 0.13% w/w, extract concentrations are 0.091 and 0.13 mg/mL respectively.

Method validation

Full description of the method's validation is provided within White 2007d (report n° J16312), which is included in this submission.

Conclusions

Total eugenol, geraniol and thymol are determined and quantified in 3AEY after dilution in methanol using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

Free eugenol, geraniol and thymol are determined and quantified in 3AEY after extraction in hexane using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

The method for quantification of eugenol in formulation was fully validated for specificity, linearity, accuracy and precision as part of study n°J16312 (White 2007d). The method is acceptable for the quantification of eugenol, geraniol and thymol (free and total active substances) in formulation 3AEY.

- **Method TS20010-1**

Comments of zRMS:	The analytical method was successfully validated for the determination of eugenol, geraniol and thymol in the plant protection product according to the requirements laid down by SANCO3030/99 rev.5.
-------------------	---

Data point:	CP 5.1.1/02 (5.2.1.1/04 of this dRR)
Report author	Wronska, L.
Report year	2020a
Report title	Validation of Analytical Method TS20010-1 for the determination of Active Ingredient Content in Cedroz and Mevalone Formulations

Report No	TS/20/010/1
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.5
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted Submitted during the renewal of approval of active substances eugenol, geraniol and thymol
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol, geraniol and thymol are determined and quantified in Mevalone after dilution in methanol using gas chromatography with a flame-ionisation detector and external standards.

Test material

Test material

Name: Mevalone
Source and lot/batch no.: Eden Research plc, batch n°11001
Active substance content: Geraniol (CAS no. 106-24-1): 6.4% w/w (64 g/kg)
Thymol (CAS no. 89-83-8): 6.4% w/w (64 g/kg)
Eugenol (CAS no. 97-53-0): 3.2% w/w (32 g/kg)
Expiry date of lot/batch: July 2022
Storage conditions: Ambient temperature

Name: Cedroz (EDN-005)
Source and lot/batch no.: Eden Research plc, batch n°12099A
Active substance content: Geraniol (CAS no. 106-24-1): 11.9% w/w (119 g/kg)
Thymol (CAS no. 89-83-8): 4.0% w/w (40 g/kg)
Expiry date of lot/batch: July 2022
Storage conditions: Ambient temperature

Reference material

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: Not state, lot n°BCCC7425, purity 99.7%
Expiry date: December 2024
Storage conditions: Not stated

Name: Geraniol
CAS number: 106-24-1
Source and lot/batch no.: Not stated, lot n°BCCC0198, purity 98.8%
Expiry date: July 2022
Storage conditions: Not stated

Name: Thymol
CAS number: 89-83-8
Source and lot/batch no.: Not stated, lot n°STBJ4851, purity 99.9%
Expiry date: October 2022
Storage conditions: Not stated

Analysis parameters

Method type: GC-FID
Instrument: H0260 Agilent GC-FID 6890

Analytical column:	ZB-5, 30 m x 0.32 mm, 0.25 µm film thickness or equivalent
Oven:	50°C (2 min) - 5°C/min – 180°C (0 min)
Split mode:	1:100
Detector temperature:	280°C
Injector temperature:	160°C
Injection volume:	1 µL
Carrier gas:	Helium
Hydrogen flow:	30 mL/min
Air Flow:	300 mL/min
N ₂ Make up Flow:	30 mL/min
Flow rate:	1.5 mL/min
Retention time:	eugenol: Approx. 19.7 min geraniol: Approx. 17 min thymol: Approx. 18 min

Sample preparation

250 mg of test item is weighed accurately into a 100 mL volumetric flask, dissolved in and adjusted to volume with methanol.

Stock solutions and calibration standards

Linearity standards are prepared in methanol.

Calibration standards are prepared in duplicate and the response factor (area/concentration) is used as confirmation that the standards were correctly prepared.

Calculations

Concentration of active substances is calculated as follows:

$$\text{Std RF} = \frac{\text{Std peak area}}{\text{Std Conc. (mg/ml)}}$$

Note: Standard concentration should include the purity.

$$w/w \% \text{ Active} = \left(\frac{\text{Peak Area Sample}}{\text{Mean Std RF}} \right) \times 100 \text{ mL} \times \left(\frac{1}{\text{Sample weight mg}} \right) \times 100 \%$$

Accuracy and repeatability

Accuracy:

Accuracy was assessed through duplicate quantification of the blank formulation fortified with active substances at three different levels.

Repeatability (precision):

Six independently prepared samples of test item were quantified according to the analytical method.

Confirmation of substance identification

Confirmation of substance identification was performed by GC-MS under conditions as close to the original quantification conditions as possible. Eugenol was successfully identified against an analytical standard.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of active substances eugenol, geraniol and thymol in plant protection product Mevalone

	Eugenol	Geraniol	Thymol
Author(s), year	Wronska, L., 2020a		

	Eugenol	Geraniol	Thymol
Principle of method	Gas chromatography with a flame-ionisation detector (GC-FID) and 1-nonanol as internal standard		
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	0.041 – 0.124 mg/mL: $y = 270.323 x + 0.287$ y = eugenol peak area, x = weight of eugenol (in mg/mL) R = 0.9985	0.080 – 0.445 mg/mL: $y = 329.998 x - 1.471$ y = geraniol peak area, x = weight of geraniol (in mg/mL) R = 0.9989	0.050 – 0.250 mg/mL: $y = 353.215 x + 0.081$ y = thymol peak area, x = weight of thymol (in mg/mL) R = 0.9989
Precision – Repeatability Mean (%RSD)	Eugenol (n = 6): 3.17% w/w (RSD: 1.05%) Horrat = 0.47	Geraniol (n = 6): 6.62% w/w (RSD: 0.70%) Horrat = 0.35	Thymol (n = 6): 6.16% w/w (RSD: 0.63%) Horrat = 0.31
Accuracy (% Recovery)	At 2.4% w/w (n = 2): 103.71% At 3.2% w/w (n = 2): 105.05% At 4.0% w/w (n = 2): 105.79%	At 4.8% w/w (n = 2): 105.94% At 6.4% w/w (n = 2): 105.94% At 8.0% w/w (n = 2): 105.77%	At 4.8% w/w (n = 2): 103.49% At 6.4% w/w (n = 2): 103.91% At 8.0% w/w (n = 2): 104.54%
Interference/ Specificity	No interferences were observed at the retention time of eugenol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.	No interferences were observed at the retention time of geraniol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.	No interferences were observed at the retention time of thymol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.
Confirmation	Confirmation of substance identification was performed by GC-MS under conditions as close to the original quantification conditions as possible. Eugenol was successfully identified against an analytical standard.	Confirmation of substance identification was performed by GC-MS under conditions as close to the original quantification conditions as possible. Geraniol was successfully identified against an analytical standard.	Confirmation of substance identification was performed by GC-MS under conditions as close to the original quantification conditions as possible. Thymol was successfully identified against an analytical standard.

Conclusion

Eugenol, geraniol and thymol is determined and quantified in Mevalone after dilution in methanol using gas chromatography with a flame-ionisation detector and external standards.

Method TS20010-1 for quantification of eugenol, geraniol and thymol in Mevalone has been demonstrated to be linear, specific, accurate and precise and SANCO/3030/99 rev.5 requirements were fulfilled.

The specificity of the analytical method was confirmed by analysis of GC-MS.

• Method for active substances release

Comments of zRMS:	Analytical method and its validation has already been evaluated during the EU approval process of the active substance.
-------------------	---

Data point:	CP 5.1.1/03 (5.2.1.1/05 of this dRR)
Report author	Kant A.
Report year	2008
Report title	Terpene Release from Encapsulated Formulation 3AEY, Appendix 4 - RFM039_ExtVal: Determination of free and encapsulated terpenes in formulation

	3AEY: Validation of extraction and GC-MS methods [Analytical phase by Kant A. 2008, RFM039_ExtrVal]
Report No	RFM039_MainPhase
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	A single ion is monitored and quantified. However, analytical grade standards are used to confirm analyte identity.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data points IIIA 2.15/01, IIIA 5.9/02, IIIA 9.10.1/01
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes

Principle of the method

The test item is diluted in tap water at a concentration of 4.08 g/L. The diluted formulation is then subjected to three separate test regimens for which specific extraction protocols from investigated substrate (liquid and solid) are presented. This summary focuses on the protocol for test item extraction from test medium.

Test material

Test material

Test Item

Name: 3AEY
Source and lot/batch no.: Eden Research plc, batch n°YPOA3Y424-1.1
Active substance content: Geraniol (CAS no. 106-24-1): 6.30% w/w (63.0 g/kg)
Thymol (CAS no. 89-83-8): 6.40% w/w (64.0 g/kg)
Eugenol (CAS no. 97-53-0): 3.21% w/w (32.1 g/kg)

Expiry date of lot/batch: not stated
Storage conditions: not stated

Reference material

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: ACROS Organics, lot n°A014510001, purity ≥ 99%
Expiry date: not stated
Storage conditions: not stated
Name: Geraniol oil
CAS number: 106-24-1
Source and lot/batch no.: Sigma-Aldrich, lot n°20431MA-476, purity ≥ 98%
Expiry date: not stated
Storage conditions: not stated

Name: Thymol
CAS number: 89-83-8
Source and lot/batch no.: Sigma-Aldrich, lot n°11915AS-111, purity ≥ 99%
Expiry date: not stated
Storage conditions: not stated

Analysis parameters

Method type: GC-MS
Instrument: Thermo Finnigan Trace-DSQ and CTC PAL autosampler
Analytical column: Zebtron ZB-5, 30 m x 0.32 mm, 1.0 µm film thickness
Oven: 50°C (2 min) - 5°C/min – 190°C (3 min)
Injector temperature: 240°C
Injection mode, flow rate: Total and free terpenes from wet systems: 1 µL Split ratio

and volume:	1:7, 1.5 mL/min Total terpenes from filter paper: 2 µL splitless, 1.2 mL/min
Carrier gas:	Helium
Transfer line:	250°C
Detection:	Electron impact
Source temperature:	200°C
Scan:	Full scan, 40 to 170 m/z ions
Monitored ions:	Eugenol: 165 m/z Geraniol: 137 m/z Thymol: 151 m/z
Retention time:	1-nonanol (Internal Standard): Approx. 20 min Eugenol from wet systems: Approx. 26 min Eugenol from dry system: Approx. 26 min Geraniol from wet systems: Approx. 23 min Geraniol from dry system: Approx. 23 min Thymol from wet systems: Approx. 24 min Thymol from dry system: Approx. 24 min

Sample preparation

The test item is diluted in tap water at a concentration of 4.08 g/L.

The diluted formulation is then subjected to three separate test regimens for which specific extraction protocols are presented.

Total active substances contents in solution:

Five replicate analyses were carried out. An aliquot (approximately 4 g) of diluted formulation was weighed into each of 5 vials (28 mL volume) containing IS2 (20 mL). The vials were shaken vigorously for 30 seconds, rolled for 30 minutes and then placed in an ultrasonic bath for 10 minutes to effect solution. The vials were then allowed to stand for 10 minutes before filtering the solvent through a Glass Micro Fibre filter into amber micro-vials for GC-MS analysis.

Free active substances contents in solution:

Approximately 4 g of diluted formulation was placed in a 15 mL conical bottomed centrifuge tube with an inert screw cap, and then centrifuged (2,000 g for 10 minutes at 15°C) to separate the solid material (YP pellet) from the aqueous phase (supernatant). The clear supernatant was carefully removed for analysis. Five replicate samples of supernatant were weighed out (approximately 4 mL) into separate vials (28 mL volume) and 20 mL of IS4 was added. The vials were gently rotated on a roller mixer for 10 minutes. The mixture was then allowed to stand for 30 minutes and the clear upper hexane layer was analysed by GC-MS.

Total active substances contents on filter paper:

Diluted formulation (0.35 mL) was applied to each of 5 pre-folded filter papers, which were then placed separately into 100 mL glass flasks. An aliquot of IS6 (20 mL) was added to each flask, which was shaken vigorously and placed on a roller board to be gently rotated for 10 minutes. The flasks were then allowed to stand for 5 minutes before approximately 2 mL of extraction solvent was transferred into an amber vial for analysis by GC-MS.

Internal standard solutions

Internal standard solution 1: 0.4 g of 1-nonanol are transferred to a 100 mL volumetric flask, dissolved in and adjusted to volume with methanol. This solution is referred to as IS1 (concentration of 4 mg/mL).

Internal standard solution 2: 10 mL of IS1 is diluted to 500 mL with methanol. This solution is referred to as IS2 (concentration of 0.08 mg/mL). IS2 was used to extract total wet system samples of 3AEY formulation at 4.08 g/L.

Internal standard solution 3: 300 mg 1-nonanol was dissolved in and adjusted to 100 mL with hexane. This solution is referred to as IS3 (concentration of 3 mg/mL).

Internal standard solution 4: 10 mL of IS3 are transferred to a 500 mL volumetric flask, diluted with and adjusted to volume with hexane to produce the internal standard solution IS4 (concentration of 0.06 mg/mL), used for sample analysis of free actives.

Internal standard solution 5: 40 mg 1-nonanol was dissolved in and adjusted to 100 mL with methanol.

This solution is referred to as IS5 (concentration of 0.4 mg/mL).

Internal standard solution 6: 10 mL of IS5 are transferred to a 500 mL volumetric flask, diluted with and adjusted to volume with methanol to produce the internal standard solution IS6 (concentration of 0.008 mg/mL), used for sample analysis of total actives on filter paper.

Stock solutions and calibration standards

Total eugenol in solution Standard Solutions: a 2 mg/mL stock is prepared by accurately weighing 200 mg eugenol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS1 and adjusted to volume with methanol to produce a calibration range over the concentration range 12 – 40 µg/mL.

Free eugenol in solution Standard Solutions: a 2.1 mg/mL stock is prepared by accurately weighing 210 mg eugenol into a 100 mL volumetric flask, dissolving in and adjusting to volume with hexane. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks and adjusted to volume with hexane to produce a calibration range over the concentration range 17 – 42 µg/mL.

Total eugenol on filter paper Standard Solutions: a 0.25 mg/mL stock is prepared by accurately weighing 25 mg eugenol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS5 and adjusted to volume with methanol to produce a calibration range over the concentration range 1 – 5 µg/mL.

Total geraniol in solution Standard Solutions: a 4 mg/mL stock is prepared by accurately weighing 400 mg geraniol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS1 and adjusted to volume with methanol to produce a calibration range over the concentration range 8 – 80 µg/mL.

Free geraniol in solution Standard Solutions: a 4 mg/mL stock is prepared by accurately weighing 360 mg geraniol into a 100 mL volumetric flask, dissolving in and adjusting to volume with hexane. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks and adjusted to volume with hexane to produce a calibration range over the concentration range 14 – 72 µg/mL.

Total geraniol on filter paper Standard Solutions: a 0.5 mg/mL stock is prepared by accurately weighing 50 mg geraniol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS5 and adjusted to volume with methanol to produce a calibration range over the concentration range 1 – 10 µg/mL.

Total thymol in solution Standard Solutions: a 4 mg/mL stock is prepared by accurately weighing 400 mg thymol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS1 and adjusted to volume with methanol to produce a calibration range over the concentration range 8 – 80 µg/mL.

Free thymol in solution Standard Solutions: a 4 mg/mL stock is prepared by accurately weighing 400 mg thymol into a 100 mL volumetric flask, dissolving in and adjusting to volume with hexane. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks and adjusted to volume with hexane to produce a calibration range over the concentration range 16 – 80 µg/mL.

Total thymol on filter paper Standard Solutions: a 0.5 mg/mL stock is prepared by accurately weighing 50 mg thymol into a 100 mL volumetric flask, dissolving in and adjusting to volume with methanol. After sonication, aliquots of this stock were transferred to 20 mL volumetric flasks containing 10 mL IS5 and adjusted to volume with methanol to produce a calibration range over the concentration range 1 – 10 µg/mL.

Calculations

Calculations were not detailed in the report.

Accuracy

To assess the accuracy of the extraction methods, 5 recovery experiments were performed using blank yeast particle (YP) formulations fortified with known concentrations (high and low) of active substances standards. Five fortifications were prepared.

Total active substances in solution:

4 g aliquots were sampled from each fortification and extracted as described above.

Free active substances in solution:

Free active substances recovery could not be determined since the “natural” free active substances level in the formulation was not known.

Total active substances on filter paper:

0.35 mL aliquots from each fortification were transferred onto filter paper and extracted as described above.

Precision

To assess the precision of the extraction methods, 5 recovery experiments were performed by preparing fresh formulation dilutions at two levels: the low level precision samples were obtained by diluting 0.8 g of formulation in 1 L of tap water, while the high level precision samples were obtained by diluting 4.5 g of formulation in 1 L of tap water. Five precision samples were prepared at each level and extracted as described above.

Total active substances in solution:

4 g aliquots were sampled from each fortification and extracted as described above.

Free active substances in solution:

4 g aliquots were sampled from each fortification and extracted as described above.

Total active substances on filter paper:

0.35 mL aliquots from each fortification were transferred onto filter paper and extracted as described above.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of active substances eugenol, geraniol and thymol release

	Eugenol	Geraniol	Thymol
Author(s), year	Kant A., 2008		
Principle of method	Gas chromatography with a mass detector (GC-MS) and 1-nonanol as internal standard		
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	<p>Total eugenol in solution: 12 – 40 µg/mL: y = 0.03x - 0.331 R= 0.9930</p> <p>Free eugenol in solution: 14 – 42 µg/mL: y = 17.519 x - 0.0019 R= 0.9894</p> <p>Total eugenol on filter paper: 1 – 5 µg/mL: y = 0.2096x - 0.2094 R= 0.9927</p> <p>y = peak area ratio (eugenol area/1-nonanol area), x = concentration of eugenol (in µg/mL)</p>	<p>Total geraniol in solution: 8 – 80 µg/mL: y = 0.0205x - 0.1728 R= 0.9958</p> <p>Free geraniol in solution: 14 – 72 µg/mL: y = 0.0201x - 0.3338 R= 0.9855</p> <p>Total geraniol on filter paper: 1 – 10 µg/mL: y = 0.1554x - 0.1196 R= 0.9981</p> <p>y = peak area ratio (geraniol area/1-nonanol area), x = concentration of geraniol (in µg/mL)</p>	<p>Total thymol in solution: 8 – 80 µg/mL: y = 0.0418x - 0.1799 R= 0.9990</p> <p>Free thymol in solution: 16 – 80 µg/mL: y = 0.0457x - 0.671 R= 0.9975</p> <p>Total thymol on filter paper: 1 – 10 µg/mL: y = 0.2431x - 0.1563 R= 0.9984</p> <p>y = peak area ratio (thymol area/1-nonanol area), x = concentration of thymol (in µg/mL)</p>
Precision – Repeatability Mean (%RSD)	<p>Total eugenol in solution at 23.03 mg/L: RSD: 4.22% (n = 5)</p> <p>Total eugenol in solution at 160.51 mg/L: RSD: 7.14% (n = 5)</p> <p>Free eugenol in solution at 16.24 mg/L: RSD: 4.67% (n = 5)</p>	<p>Total geraniol in solution at 54.31 mg/L: RSD: 4.11% (n = 5)</p> <p>Total geraniol in solution at 281.25 mg/L: RSD: 2.82% (n = 5)</p> <p>Free geraniol in solution at 38.31 mg/L: RSD: 4.43% (n = 5)</p>	<p>Total thymol in solution at 54.72 mg/L: RSD: 3.48% (n = 5)</p> <p>Total thymol in solution at 317.94 mg/L: RSD: 4.34% (n = 5)</p> <p>Free thymol in solution at 41.60 mg/L: RSD: 5.64% (n = 5)</p>

	Eugenol	Geraniol	Thymol
	<p>Free eugenol in solution at 120.36 mg/L: RSD: 2.56% (n = 5)</p> <p>Total eugenol on paper filter at 10.81 µg/filter paper: RSD: 3.62% (n = 5)</p> <p>Total eugenol on paper filter at 46.67 µg/filter paper: RSD: 5.39% (n = 5)</p>	<p>Free geraniol in solution at 252.16 mg/L: RSD: 2.25% (n = 5)</p> <p>Total geraniol on paper filter at 19.78 µg/filter paper: RSD: 5.53% (n = 5)</p> <p>Total geraniol on paper filter at 85.73 µg/filter paper: RSD: 3.73% (n = 5)</p>	<p>Free thymol in solution at 288.84 mg/L: RSD: 1.32% (n = 5)</p> <p>Total thymol on paper filter at 19.30 µg/filter paper: RSD: 4.69% (n = 5)</p> <p>Total thymol on paper filter at 99.24 µg/filter paper: RSD: 8.54% (n = 5)</p>
Accuracy (% Recovery)	<p>Total eugenol in solution at 24.5 mg/mL: 92.35% (n = 5) RSD: 12.71%</p> <p>Total eugenol in solution at 150.8 mg/mL: 95.56% (n = 5) RSD: 10.57%</p> <p>Total eugenol on filter paper at 10.01 µg/filter paper: 105.6% (n = 5) RSD: 9.19%</p> <p>Total eugenol on filter paper at 52.89 µg/filter paper: 93.20% (n = 5) RSD: 6.05%</p>	<p>Total geraniol in solution at 26.1 mg/mL: 88.88% (n = 5) RSD: 5.01%</p> <p>Total geraniol in solution at 301.3 mg/mL: 96.43% (n = 5) RSD: 7.76%</p> <p>Total geraniol on filter paper at 10.12 µg/filter paper: 109.35% (n = 5) RSD: 7.78%</p> <p>Total geraniol on filter paper at 105.88 µg/filter paper: 94.43% (n = 5) RSD: 3.46%</p>	<p>Total thymol in solution at 24.7 mg/mL: 84.70% (n = 5) RSD: 6.12%</p> <p>Total thymol in solution at 300.6 mg/mL: 95.62% (n = 5) RSD: 6.74%</p> <p>Total thymol on filter paper at 9.35 µg/filter paper: 94.40% (n = 5) RSD: 6.42%</p> <p>Total thymol on filter paper at 104.72 µg/filter paper: 99.81% (n = 5) RSD: 3.98%</p>
Interference/ Specificity	<p>No interferences were observed at the retention time of eugenol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.</p>	<p>No interferences were observed at the retention time of geraniol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.</p>	<p>No interferences were observed at the retention time of thymol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.</p>
LOQ	<p>24.5 mg/L for total eugenol in aqueous solution 16.24 mg/L for free eugenol in aqueous solution 10.00 µg/filter paper for total eugenol on filter paper</p>	<p>26.1 mg/L for total geraniol in aqueous solution 38.31 mg/L for free geraniol in aqueous solution 10.12 µg/filter paper for total geraniol on filter paper</p>	<p>24.7 mg/L for total thymol in aqueous solution 41.60 mg/L for free thymol in aqueous solution 9.35 µg/filter paper for total thymol on filter paper</p>
LOD	<p>2.50 mg/L for total eugenol in aqueous solution 3.00 mg/L for free eugenol in aqueous solution 0.50 µg/filter paper for total eugenol on filter paper</p>	<p>2.50 mg/L for total geraniol in aqueous solution 3.00 mg/L for free geraniol in aqueous solution 0.50 µg/filter paper for total geraniol on filter paper</p>	<p>1.25 mg/L for total thymol in aqueous solution 1.50 mg/L for free thymol in aqueous solution 0.25 µg/filter paper for total thymol on filter paper</p>

Conclusion

Total and free eugenol, geraniol and thymol from diluted 3AEY formulation is quantified by GC-MS with internal standard following extraction from aqueous solutions or dry filter paper deposits.

Verification of linearity, accuracy, precision and specificity demonstrated the method is acceptable for quantification of total and free thymol from dilute 3AEY formulation. The method is acceptable for the quantification of eugenol, geraniol and thymol with respective Limit of Quantification of 24.5 mg/L (total eugenol), 26.1 mg/L (total geraniol) and 24.7 mg/L (total thymol) in aqueous solution, 16.24 mg/L (free eugenol), 38.31 mg/L (free geraniol) and 41.60 mg/L (free thymol) in aqueous solution, 10.00 µg/filter paper (total eugenol), 10.12 µg/filter paper (total geraniol) and 9.35 µg/filter paper (total thymol) on filter paper.

Although the work is not conducted to GLP, care was taken to provide detailed description of all the procedures. The analytical methods presented can be relied upon.

Comments of zRMS:	Analytical method and its validation has already been evaluated during the EU approval process of the active substance.
-------------------	---

Data point:	CP 5.1.1/04 (5.2.1.1/06 of this dRR)
Report author	Kant A.
Report year	2008
Report title	Terpene Release from Encapsulated Formulation 3AEY, Appendix 5 - RFM039_APcIMSval: Validation of the APcI-MS Headspace Analysis Method [Analytical phase by Kant A. 2008, APcIMSval]
Report No	RFM039_MainPhase
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	A single ion is monitored and quantified. However, analytical grade standards are used to confirm analyte identity.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 2.15/01, IIIA 5.9/01, IIIA 9.10.1/01
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes

Principle of the method

The test item is diluted in tap water at a concentration of 4.08 g/L. The diluted formulation is then subjected to three separate test regimens in enclosed headspace-sampling test vessels. Release of eugenol into the headspace of sampling vessels was quantified by atmospheric pressure chemical ionisation-mass spectrometry (APcI-MS). This summary focuses on the protocol for test item extraction from test medium.

Test material

Test material

Test Item	
Name:	3AEY
Source and lot/batch no.:	Eden Research plc, batch n°YPOA3Y424-1.1
Active substance content:	Geraniol (CAS no. 106-24-1): 6.30% w/w (63.0 g/kg) Thymol (CAS no. 89-83-8): 6.40% w/w (64.0 g/kg) Eugenol (CAS no. 97-53-0): 3.21% w/w (32.1 g/kg)
Expiry date of lot/batch:	not stated
Storage conditions:	not stated

Reference material

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	ACROS Organics, lot n°A014510001, purity ≥ 99%
Expiry date:	not stated
Storage conditions:	not stated

Name: Geraniol oil
CAS number: 106-24-1
Source and lot/batch no.: Sigma-Aldrich, lot n°20431MA-476, purity \geq 98%
Expiry date: not stated
Storage conditions: not stated

Name: Thymol
CAS number: 89-83-8
Source and lot/batch no.: Sigma-Aldrich, lot n°11915AS-111, purity \geq 99%
Expiry date: not stated
Storage conditions: not stated

Analysis parameters

Method type: APcI-MS
Instrument: Micromass Platform LCZ mass spectrometer connected to a custom-built headspace sampling interface
Analytical column: There is no chromatographic column used for separating components.
APcI mode: Positive
Transfer line temperature: 175°C
Interface temperature: 50°C
Source block temperature: 50°C
Carrier gas: Nitrogen 10 mL/min
Sampling flow rate: 10 mL/min
Cone voltage: 12 V
Capillary voltage: 4 V
Base ion monitored: Geraniol: 137 m/z
Thymol: 151 m/z
Eugenol: 165 m/z

Sample preparation

The test item is diluted in tap water at a concentration of 4.08 g/L.

The diluted formulation is then used to either fortify water to simulate spraying over water, or fortify a filter paper that is then either let to dry or subject to a wet-dry-wet procedure.

Release of geraniol, eugenol and thymol was measured in three replicate vessels containing an aqueous 3AEY suspension (15 mL at 4.08 g product/L of tap water) at room temperature. Pre-equilibration headspace sampling was performed at 0, 20, 40, 60 and 80 minutes. Once headspace terpene levels were stable, a flow of air (60 mL/min) was introduced, gentle stirring commenced (60 rpm using a magnetic stir bar) and APcI-MS sampling was initiated. Headspace measurements were recorded every hour during the first 6 hours, then at 24 hours, and at 24-hourly intervals for up to 168 hours (7 days, no measurement was taken at 144 hours/Day 6), or until headspace concentrations of geraniol, eugenol and thymol were below the limit of detection.

Based on the procedure, no extraction took place. Direct injection of headspace samples into a mass spectrometer provided quantification of evaporated eugenol, geraniol and thymol.

Stock solutions and calibration standards

Blank formulation matrix: 4.08 g of blank formulation (yeast cells suspension containing no active ingredient) was diluted to 1 L with water.

A stock solution containing 160 mg/L eugenol, 280 mg/L geraniol and 300 mg/L thymol in water was prepared. This stock solution was serially diluted in the blank formulation matrix to produce a series of calibration standards.

Compensation of MS signal drift:

To compensate for mass spectrometer signal drift over time, instrument calibration was confirmed on each day of analysis, which required the generation of gas phase terpenes from standards of known geraniol, eugenol and thymol concentration. Standards were prepared as three individual hexane solutions at concentrations that would register a signal intensity equivalent to 100 ppbv when sampling 75 mL of headspace volume. Individual stock solutions of geraniol, eugenol and thymol (3.44, 3.65 and 3.35 mg/mL, respectively) were prepared by mixing aliquots (34.4, 36.5 and 33.5 mg, respectively) of each terpene with hexane to a final volume of 10 mL. Terpene standards for APcI-MS calibration were prepared by diluting aliquots (100 µL) of each stock solution in hexane to a final volume of 10 mL.

Each terpene standard was introduced into the nitrogen make-up gas using a syringe pump at a flow rate of 1.5 µL/min (Harvard Apparatus) and volatilised before entering the MS source. The signal intensity (peak height) produced was related to the amount of each volatile terpene entering the source (51.6, 54.9 and 50.3 ng/min for geraniol, eugenol and thymol, respectively). A comparison of 3AEY standard peak heights with those obtained from individual samples prepared with known concentrations of geraniol, eugenol and thymol allowed an estimation of the maximum amount of each terpene (ng/min) entering the source, and these values were expressed as parts per billion in the gas phase (ppbv).

Calculations

Calculations were not detailed in the report.

Accuracy, precision

Accuracy could not be determined since no extraction took place or sample clean-up. Instead of the usual procedure, mass balance on the samples was provided by extraction of the substrate and quantification by GC-MS, as well as verification of calibration standards linearity. Precision was assessed through the quintuplicate analysis of calibration standards.

Accuracy, precision:

Wet system: It is not possible to assess the accuracy of APcI-MS measurements in the traditional manner, as equipment calibration is performed using the same technique of headspace sampling at a pre-determined timepoint without extraction or sample clean-up of volatiles. In order to assess linearity, precision and accuracy of headspace sampling, APcI-MS measurements of samples containing a mixture of blank matrix and a known concentration of each terpene (see calibration standards) were recorded on 5 separate occasions in a random order.

The total and free amount of active substances in the test solutions was quantified by GC-MS prior to addition to the test system, and again at the end of the test. The overall loss from the test substrate was then calculated.

	Eugenol wet system	Geraniol wet system	Thymol wet system
Nominal (mg/L)	140.02	245.85	235.26
Aq. Phase (free, mg/L)	103.76	167.31	165.84
Encapsulated (mg/L)	36.26	78.54	69.42
Recovered from aqueous solution (mg/L)	< 25.05	0	0
Total recovered (mg/L)	> 114.97	245.85	235.26
Mass balance	> 82%	100%	100%

Dry system: the total amount of active substances extracted from the filter paper was quantified by GC-MS and was compared to the nominal amount. The overall loss of test substance was then calculated.

	Eugenol dry system	Geraniol dry system	Thymol dry system
Nominal (µg)	41.44	81.31	85.07
Final amount on filter paper at 72 hrs (total, µg)	16.48	15.58	18.18
Released to headspace (µg)	24.96	65.73	66.89
Total evaporated (%)	60%	81%	79%

Dry-wet-dry system: the total amount of active substances extracted from the filter paper was quantified by GC-MS and was compared to the nominal amount. The overall loss of test substances was then calculated.

	Eugenol Dry-wet-dry system	Geraniol Dry-wet-dry system	Thymol Dry-wet-dry system
Nominal (µg)	52.22	94.99	90.59
Final amount on filter paper at 72 hrs (total, µg)	< 10.01	0.0	0.0
Released to headspace (µg)	42.21	94.99	90.59
Total evaporated (%)	> 81%	100%	100%

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of active substances eugenol, geraniol and thymol release

	Eugenol	Geraniol	Thymol
Author(s), year	Kant A., 2008		
Principle of method	Atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS)		
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	<p>Eugenol in wet system: 2.5 – 160 mg/L: y = 10.314x + 9.7616 R= 0.9999</p> <p>Eugenol in dry system: 10 – 160 µg/L: y = 6.0901x + 3.0952 R= 0.9963</p> <p>y = headspace eugenol concentration (ppbv), x = concentration of eugenol in the 4.08 g/L YPs sample (in mg/L)</p>	<p>Geraniol in wet system: 5.31 – 280 mg/L: y = 18.51x + 15.37 R= 0.9999</p> <p>Geraniol in dry system: 21.25 to 280 µg/L: y = 10.484x – 44.425 R= 0.9976</p> <p>y = headspace geraniol concentration (ppbv), x = concentration of geraniol in the 4.08 g/L YPs sample (in mg/L)</p>	<p>Thymol in wet system: 4.69 – 300 mg/L: y = 17.786x – 51.456 R= 0.9997</p> <p>Thymol in dry system: 18.75 – 300 µg/L: y = 8.352x – 59.502 R= 0.9978</p> <p>y = headspace thymol concentration (ppbv), x = concentration of thymol in the 4.08 g/L YPs sample (in mg/L))</p>
Precision – Repeatability Mean (%RSD)	<p>Eugenol headspace concentration: For a 280 mg/L standard solution: 1 777 ppbv RSD: 3.26% (n = 5)</p> <p>For a 140 mg/L standard solution: 899 ppbv RSD: 1.34% (n = 5)</p> <p>For a 70 mg/L standard solution: 466 ppbv RSD: 5.38% (n = 5)</p> <p>For a 37.5 mg/L standard solution: 233 ppbv RSD: 2.45% (n = 5)</p> <p>For a 17.5 mg/L standard solution: 116 ppbv RSD: 6.04% (n = 5)</p> <p>For a 8.75 mg/L standard solution: 64 ppbv RSD: 1.94% (n = 5)</p> <p>For a 4.38 mg/L standard solution: 33 ppbv RSD: 4.62% (n = 5)</p>	<p>Geraniol headspace concentration: For a 280 mg/L standard solution: 5 458 ppbv RSD: 3.39% (n = 5)</p> <p>For a 140 mg/L standard solution: 2 764ppbv RSD: 1.68% (n = 5)</p> <p>For a 70 mg/L standard solution: 1 399 ppbv RSD: 4.92% (n = 5)</p> <p>For a 35 mg/L standard solution: 692 ppbv RSD: 4.44% (n = 5)</p> <p>For a 17.5 mg/L standard solution: 339 ppbv RSD: 5.56% (n = 5)</p> <p>For a 8.75 mg/L standard solution: 187 ppbv RSD: 1.81% (n = 5)</p> <p>For a 4.38 mg/L standard solution: 100 ppbv RSD: 4.34% (n = 5)</p>	<p>Thymol headspace concentration: For a 300 mg/L standard solution: 5 469 ppbv RSD: 3.26% (n = 5)</p> <p>For a 150 mg/L standard solution: 2 612 ppbv RSD: 2.01% (n = 5)</p> <p>For a 75 mg/L standard solution: 1 266 ppbv RSD: 4.14% (n = 5)</p> <p>For a 37.5 mg/L standard solution: 613 ppbv RSD: 4.19% (n = 5)</p> <p>For a 18.75 mg/L standard solution: 290 ppbv RSD: 6.41% (n = 5)</p> <p>For a 9.38 mg/L standard solution: 159 ppbv RSD: 2.24% (n = 5)</p> <p>For a 4.70 mg/L standard solution: 83 ppbv RSD: 4.21% (n = 5)</p>
Interference/ Specificity	The optimal ion for eugenol was the 165 m/z, however, it was found that thymol also	Optimal ions for APCI-MS analysis were produced at 137 m/z for geraniol. Based	Optimal ions for APCI-MS analysis were produced at 151 m/z for thymol. Based

	Eugenol	Geraniol	Thymol
	produces an ion at 165 m/z and therefore thymol interfered with eugenol. As the level of interference was constant (approximately 15%), eugenol concentrations were corrected for thymol interference. Analyte specificity was also confirmed via the use of analytical grade standards.	on individual and comparative headspace analysis, no interference at or above 3% of the signal was observed for geraniol. Analyte specificity was also confirmed via the use of analytical grade standards.	on individual and comparative headspace analysis, no interference at or above 3% of the signal was observed for thymol. Analyte specificity was also confirmed via the use of analytical grade standards.
LOQ	64 ppbv (equivalent to 5.0 mg/L)	187 ppbv (equivalent to 10.625 mg/L)	159 ppbv (equivalent to 9.375 mg/L)
LOD	31.1 ppbv (equivalent to 4.375 mg/L)	93.7 ppbv (equivalent to 5.313 mg/L)	81 ppbv (equivalent to 4.688 mg/L)

Conclusion

The rate of evaporation of active substances from solid and aqueous substrates is quantified using atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS). The method involves direct sampling of the headspace above the substrate and does not include chromatographic separation of the different analytes.

Accuracy could not be verified since no extraction is taking place; however, linearity of the method was confirmed through determination of headspace concentration of samples of known active substances concentration. In addition, precision was assessed through five independent quantifications of the analytical standards, analysed in a random order. %RSD was acceptable in each case. The method is acceptable for the quantification of eugenol, geraniol and thymol with respective Limits of Quantification of 64 ppbv for eugenol, 187 ppbv for geraniol and 159 ppbv for thymol in headspace.

Although the work is not conducted to GLP, care was taken to provide detailed description of all the procedures. The analytical methods presented can be relied upon.

5.2.1.2 Description of analytical methods for the determination of relevant impurities (KCP 5.1.1)

Methyleugenol is a relevant impurity in technical grade eugenol. It is present at a maximum limit of 1 g/kg. The following analytical methods for the determination of methyleugenol in the plant protection product Mevalone have previously been reviewed (within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU) and are provided in support of this assessment:

- analytical method M737 for methyleugenol determination in Mevalone
- analytical method TS21001-1 for methyleugenol determination in Mevalone

• Method M737

Comments of zRMS:	The analytical method M373 was successfully validated for the quantification of the methyleugenol in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4. Validation complies with SANCO/3030/99 rev.5.
-------------------	--

Data point:	CP 5.1.1/05 (5.2.1.2/01 of this dRR)
Report author	Bates, G.J.D.
Report year	2012a
Report title	Validation of Analytical Method M737, Gas chromatographic determination of methyl

	eugenol in formulations, for low levels of methyl eugenol in the 3AEY formulation
Report No	J19055
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (Addendum 2012) under data point B5.1.3.2
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The impurity methyleugenol is determined and quantified using gas chromatography with a mass detector and 1-nonanol as internal standard. The method used is referenced as M737.

Test material

Test material

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, batch n°YPOA3Y424-1.1 Geraniol (CAS no. 106-24-1): 6.61% w/w (66.1 g/kg) Thymol (CAS no. 89-83-8): 6.69% w/w (66.9 g/kg) Eugenol (CAS no. 97-53-0): 3.59% w/w (35.9 g/kg)
Expiry date of lot/batch:	not stated
Storage conditions:	not stated

Reference material

Name:	Eugenol Methyl Ether (methyleugenol)
CAS number:	93-15-2
Source and lot/batch no.:	Sigma-Aldrich, lot n°46110, purity 99.2%
Expiry date:	not specified
Storage conditions:	In a refrigerated dessicator

Analysis parameters

Method type	GC-MS
Instrument:	GC, 5890 (Hewlett Packard) with a 5970 Series MSD
Analytical column:	Solgel-wax, 30 m x 0.25 mm, 0.25 µm film thickness or equivalent
Oven:	50°C (2 min) - 15°C/min – 240°C (2 min)
Detector temperature:	280°C
Injector temperature:	250°C
Injection volume:	5.0 µL
Injection mode:	Split 20:1 using SGE Focusliner
Carrier gas:	Helium
Flow rate:	1.0 mL/min
Acquisition type:	SIM
Retention time:	1-nonanol (Internal Standard): Approx. 7.0 min Methyleugenol: Approx. 9.6 min
Monitored ions:	1-nonanol (Internal Standard): 97 m/z Methyleugenol: 178 m/z

Sample preparation

Two separate samples are prepared, each in duplicate: 4 to 6 g test item is weighed into separate 125 mL screw-topped flasks. The diluted Internal standard solution (25 mg/L), 20 mL is added, the flask is capped, shaken and sonicated 10 min to ensure complete dissolution of the sample. The sample is filtered

through a GFC filter and quantified by GC-MSD. Based on a nominal methyleugenol content of 7 mg/kg, the sample concentrations are 1.4 to 2.1 µg/mL.

Stock solutions and calibration standards

- Internal standard solutions:

0.25 g of 1-nonanol are transferred to a 1L volumetric flask, dissolved in and adjusted to volume with methanol. This solution is referred to as IS1. Then this solution is diluted 10 times to obtain a Dilute Internal Standard (for example 20 mL to 200 mL with methanol).

- Methyleugenol standard solutions:

For an expected methyleugenol content of 7 mg/kg in the test item, analytical grade methyleugenol (0.05 and 0.1 g) is weighed into a 100 mL volumetric flask, made up to volume with methanol and shaken thoroughly to mix. Then these solutions are diluted 10 times to obtain a Solution A and Solution B (containing respectively 10 and 5 µg/mL of methyleugenol). 10 mL of Solution B is diluted 10 times to obtain a Solution C (0.5 µg/mL of methyleugenol).

Working standard solution are prepared using 3 mL of Solution A and 20 mL of Solution C to individual 100 mL flasks, then diluted with 10 mL of Dilute Internal Standard solution and make up with methanol. The calibration range spans concentrations from 0.2 to 5 µg/mL of methyleugenol. Six calibration standards are prepared.

Calculations

The same calculations are applied to calculate methyleugenol content.

Concentration of methyleugenol is calculated as follows:

$$\text{Impurity content} = \frac{\text{AE}}{\text{AIS}} \times \frac{\text{Wt IS} \times 1\,000\,000}{\text{Wts} \times \text{FAv.}}$$

Where:

- Impurity content = methyleugenol content in mg/kg
- AE = area methyleugenol peak in sample
- AIS = area Internal Standard peak in sample
- Wt IS = Weight of Dilute Internal Standard solution
- Wts = Weight of sample taken
- FAv. = average Response Factor from the internal standards before and after the sample

Accuracy and repeatability

Three recovery experiments were carried out in which a sample of the supplied 3AEY formulation were analysed alone and then re-analysed fortified with the methyleugenol at three different concentrations.

The recoveries were then analysed, according to the method, and the amounts of the impurity were calculated.

Repeatability (precision):

Six replicate samples of the 3AEY formulation were weighed out. The weights covered the range of weight specified in the method, about ± 25% of the nominal weight, and were analysed.

Confirmation of substance identification

Confirmation of substance identification was performed by GC-MS under conditions as close to the original quantification conditions as possible. Methyleugenol was successfully identified against an analytical standard.

Validation - Results and discussions

Table 5.2-3: Method suitable for the determination of impurity methyleugenol in plant protection product Mevalone

	Methyleugenol
Author(s), year	Bates G.J.D., 2012

	Methyleugenol
Principle of method	Gas chromatography with a mass detector (GC-MS) and 1-nonanol as internal standard
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	0.20 – 5.00 µg/mL: $y = 2364.1756 x - 0.0101$ y = peak area ratio (methyleugenol area/1-nonanol area), x = weight of methyleugenol (in g) $R \geq 0.9996$
Precision – Repeatability Mean (%RSD)	Methyleugenol (n = 6): 3.42 mg/kg (RSD: 0.37%) Horrat = 0.04
Accuracy (% Recovery)	At 0.98 mg/kg (n = 2): 100.6% At 4.8 mg/kg (n = 2): 102.2% At 9.88 mg/kg (n = 2): 100.8%
Interference/ Specificity	No interferences were observed at the retention time of methyleugenol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.
LOQ LOD	1 mg/kg 0.2 mg/kg
Confirmation	The retention times and mass spectra for the peaks eluting at the retention times ascribed to methyleugenol were compared for both the standard and test item sample injections.

Conclusions

Methyleugenol impurity is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a mass detector 1-nonanol as internal standard.

The analytical method M737 for quantification of methyleugenol in 3AEY has been demonstrated to be linear, specific, accurate and precise and SANCO/3029/99 rev.4 requirements were fulfilled. In addition, the method also complies with requirements according to SANCO/3030/99 rev.5. The method is acceptable for the quantification of methyleugenol in formulation 3AEY with a Limit of Quantification of 1 mg/kg.

The specificity of the analytical method was confirmed by analysis of GC-MS.

Comments of zRMS:	The analytical method M737 was successfully validated for the quantification of Methyleugenol in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4.
-------------------	--

Data point:	CP 5.1.2/17 (5.2.1.2/02 of this dRR)
Report author	Bates, G.J.D.
Report year	2012b
Report title	Accelerated Storage Stability Trial For The 3AEY Formulation – Amended report
Report No	J19052
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The impurity methyleugenol is determined and quantified using gas chromatography with a mass detector and 1-nonanol as internal standard. The method used is referenced as M737. The method is fully

summarised within the study Bates 2012b (report n°J19055), included in this submission.

Test material

Test material

Test Item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, batch n°53167-022 Geraniol (CAS no. 106-24-1): 6.49% w/w (64.9 g/kg) Thymol (CAS no. 89-83-8): 6.78% w/w (67.8 g/kg) Eugenol (CAS no. 97-53-0): 3.47% w/w (34.7 g/kg)
Expiry date of lot/batch:	not stated
Storage conditions:	not stated

Reference material

Name:	Eugenol Methyl Ether (methyleugenol)
CAS number:	93-15-2
Source and lot/batch no.:	Sigma-Aldrich, lot n°46110, purity 99.2%
Expiry date:	not specified
Storage conditions:	In a refrigerated dessicator

Analysis parameters

Method type	GC-MS
Instrument:	GC, 5890 (Hewlett Packard) with a 5970 Series MSD
Analytical column:	Solgel-wax, 30 m x 0.25 mm, 0.25 µm film thickness or equivalent
Oven:	50°C (2 min) - 15°C/min – 240°C (2 min)
Detector temperature:	280°C
Injector temperature:	250°C
Injection volume:	5.0 µL
Injection mode:	Split 20:1 using SGE Focusliner
Carrier gas:	Helium
Flow rate:	1.0 mL/min
Acquisition type:	SIM
Retention time:	1-nonanol (Internal Standard): Approx. 7.0 min Methyleugenol: Approx. 9.6 min
Monitored ions:	1-nonanol (Internal Standard): 97 m/z Methyleugenol: 178 m/z

Sample preparation

Two separate samples are prepared, each in duplicate: 4 to 6 g test item is weighed into separate 125 mL screw-topped flasks. The diluted Internal standard solution(25 mg/L), 20 mL is added, the flask is capped, shaken and sonicated 10 min to ensure complete dissolution of the sample. The sample is filtered through a GFC filter and quantified by GC-MSD. Based on a nominal methyleugenol content of 7 mg/kg, the sample concentrations are 1.4 to 2.1 ng/mL.

Method validation

Full description of the method's validation is provided within Bates 2012b (report n°J19055), which is included in this submission.

Conclusions

Methyleugenol impurity is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a mass detector 1-nonanol as internal standard.

The method for quantification of eugenol in formulation was fully validated for specificity, linearity, accuracy and precision as part of study n°J19055 (Bates 2012b). The method is acceptable for the quantification of methyleugenol in formulation 3AEY with a Limit of Quantification of 1 mg/kg.

Comments of zRMS:	The analytical method M737 was successfully validated for the determination of the
-------------------	--

	Methyl-eugenol impurity in the plant protection product according to the requirements laid down by SANCO3030/99 rev.4.
--	--

Data point:	CP 5.1.2/18
Report author	Bates, G.J.D.
Report year	2014
Report title	Ambient Storage Stability Trial For The 3AEY Formulation
Report No	J19053
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The impurity methyleugenol is determined and quantified using gas chromatography with a mass detector and 1-nonanol as internal standard. The method used is referenced as M737. The method is fully summarised within the study Bates 2012b (report n°J19055), included in this submission.

Test material

Test material

Test Item	
Name:	3AEY
Source and lot/batch no.:	Eden Research plc, batch n°53167-022
	Geraniol (CAS no. 106-24-1): 6.49% w/w (64.9 g/kg)
	Thymol (CAS no. 89-83-8): 6.78% w/w (67.8 g/kg)
	Eugenol (CAS no. 97-53-0): 3.47% w/w (34.7 g/kg)
Expiry date of lot/batch:	not stated
Storage conditions:	not stated

Reference material

Name:	Eugenol Methyl Ether (methyleugenol)
CAS number:	93-15-2
Source and lot/batch no.:	Sigma-Aldrich, lot n°46110, purity 99.2%
Expiry date:	not specified
Storage conditions:	In a refrigerated dessicator

Analysis parameters

Method type	GC-MS
Instrument:	GC, 5890 (Hewlett Packard) with a 5970 Series MSD
Analytical column:	Solgel-wax, 30 m x 0.25 mm, 0.25 µm film thickness or equivalent
Oven:	50°C (2 min) - 15°C/min – 240°C (2 min)
Detector temperature:	280°C
Injector temperature:	250°C
Injection volume:	5.0 µL
Injection mode:	Split 20:1 using SGE Focusliner
Carrier gas:	Helium
Flow rate:	1.0 mL/min
Acquisition type:	SIM
Retention time:	1-nonanol (Internal Standard): Approx. 7.0 min

Monitored ions: Methyleugenol: Approx. 9.6 min
1-nonanol (Internal Standard): 97 m/z
Methyleugenol: 178 m/z

Sample preparation

Two separate samples are prepared, each in duplicate: 4 to 6 g test item is weighed into separate 125 mL screw-topped flasks. The diluted Internal standard solution(25 mg/L), 20 mL is added, the flask is capped, shaken and sonicated 10 min to ensure complete dissolution of the sample. The sample is filtered through a GFC filter and quantified by GC-MSD. Based on a nominal methyleugenol content of 7 mg/kg, the sample concentrations are 1.4 to 2.1 ng/mL.

Method validation

Full description of the method's validation is provided within Bates 2012b (report n°J19055), which is included in this submission.

Example chromatograms of samples and calibration standards are presented in the report.

Conclusions

Methyleugenol impurity is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a mass detector 1-nonanol as internal standard.

The method for quantification of eugenol in formulation was fully validated for specificity, linearity, accuracy and precision as part of study n°J19055 (Bates 2012b). The method is acceptable for the quantification of methyleugenol in formulation 3AEY with a Limit of Quantification of 1 mg/kg.

- **Method TS20010-1**

Comments of zRMS:	The analytical method was successfully validated for the determination of Methyl-Eugenol in the plant protection product according to the requirements laid down by SANCO3030/99 rev.5.
-------------------	---

Data point:	CP 5.1.1/06 (5.2.1.2/03 of this dRR)
Report author	Wronska, L.
Report year	2020b
Report title	Validation of an Analytical Method TS21001-1 for the determination of Impurity Methyl-Eugenol in Mevalone Formulations
Report No	TS/21/001/1
Document No	Not applicable
Guidelines followed in study	SANCO/3030/99 rev.5
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Methyleugenol is determined and quantified in Mevalone after dilution in methanol using gas chromatography with a MS detector and external standards.

Test material

Test material

Name: Mevalone/3AEY/EDN-004
Source and lot/batch no.: Eden Research plc, batch n°11001
Active substance content: Geraniol (CAS no. 106-24-1): 6.4% w/w (64 g/kg)

Thymol (CAS no. 89-83-8): 6.4% w/w (64 g/kg)
Eugenol (CAS no. 97-53-0): 3.2% w/w (32 g/kg)
Expiry date of lot/batch: July 2022
Storage conditions: Ambient temperature

Reference material

Name: Methyleugenol
CAS number: 93-15-2
Source and lot/batch no.: Not stated, lot n°BCCC0277, purity 99.0%
Expiry date: July 2022
Storage conditions: Not stated

Name: Thymol
CAS number: 89-83-8
Source and lot/batch no.: Not stated, lot n°STBJ4851, purity 99.9%
Expiry date: October 2022
Storage conditions: Not stated

Name: Eugenol
CAS number: 97-53-0
Source and lot/batch no.: Not state, lot n°BCCC7425, purity 99.7%
Expiry date: December 2024
Storage conditions: Not stated

Name: Geraniol
CAS number: 106-24-1
Source and lot/batch no.: Not stated, lot n°BCCC0198, purity 98.8%
Expiry date: July 2022
Storage conditions: Not stated

Analysis parameters

Method type: GC-MS
Instrument: Agilent H0510 Agilent GC-MS 7890-5975
Analytical column: ZB-5, 30 m x 0.32 mm, 0.25 µm film thickness or equivalent
Oven: 50°C (2 min) - 5°C/min – 180°C (0 min)
Injector temperature: 160°C
Injection volume: 1.0 µL
Injection mode: Split 10:1
Carrier gas: Helium
Flow rate: 1.5 mL/min
MS transfer Line: 250°C
EI Source temperature: 230°C
Quad temperature: 150°C
Acquisition type: SIM
Retention time: Methyleugenol: Approx. 19.4 min
Monitored ions: Methyleugenol: 177, 178 and 179 m/z

Sample preparation

5 g of test item is weighed accurately into a 20 mL volumetric flask, dissolved with 15 mL methanol and disperse gently by swirling (not shake). The sample is sonicated and filtered through a 0.45 µm filter into vials. For a nominal content of 33.0 mg/kg methyleugenol in Mevalone, methyleugenol concentration in the sample is 11.0 µg/mL.

Stock solutions and calibration standards

40 mg of methyleugenol standard is weighed accurately into a 20 mL volumetric flask, then made up to the volume with methanol. The concentration in the solution Stock A1 is 2000 µg/mL.

5 mL of Stock A1 is diluted into 50 mL of methanol and label as Stock A2 (200 µg/mL).
10 mL of Stock A2 is diluted into 100 mL of methanol and label as Stock A3 (20 µg/mL).
These solutions are diluted in methanol to obtain 5 linearity standards covering the range 4.0 to 40.0 µg/mL.

Calculations

$$w/w \% \text{ impurity} = \left(\frac{\text{Peak Area Sample} - c}{m} \right) \times 20 \text{ mL} \times \left(\frac{1}{\text{Sample weight mg}} \right) \times 100 \%$$

Where: c is the intercept and m is the slope.

Accuracy and repeatability

Accuracy was assessed through quantification of the blank formulation fortified with methyleugenol at three different levels. Fortification was performed at 16.8 mg/kg, 33.2 mg/kg and 186.6 mg/kg. Samples were prepared as described above and produced concentrations of 5.6, 11.6 and 62.2 µg/mL.

Repeatability (precision):

Six independently prepared samples of test item were quantified according to the analytical method.

Validation - Results and discussions

Table 5.2-4: Method suitable for the determination of impurity methyleugenol in plant protection product Mevalone

	Methyleugenol
Author(s), year	Bates G.J.D., 2012
Principle of method	Gas chromatography with a mass detector (GC-MS) and 1-nonanol as internal standard
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	4.20 to 46.64 µg/mL: y = 204.36 x + 88.646 y = methyleugenol peak area, x = content of methyleugenol (in µg/mL) R = 0.9992
Precision – Repeatability Mean (%RSD)	Methyleugenol (n = 6): 19.6 mg/kg (RSD: 6.92%) Horrat = 1
Accuracy (% Recovery)	At 16.8 mg/kg (n = 2): 116.2% At 33.2 mg/kg (n = 2): 103.4% At 186.6 mg/kg (n = 2): 100.8%
Interference/ Specificity	No interferences were observed at the retention time of methyleugenol or the internal standard. Example chromatograms of samples and calibration standards are presented in the report.
LOQ	16.8 mg/kg
Confirmation	The retention times and mass spectra for the peaks eluting at the retention times ascribed to methyleugenol were compared for both the standard and test item sample injections.

Conclusion

Methyleugenol is determined and quantified in Mevalone after dilution in methanol using gas chromatography with a MS detector and external standards.

Method TS21001-1 for quantification of methyleugenol in Mevalone has been demonstrated to be linear, specific, accurate and precise and SANCO/3030/99 rev.5 requirements were fulfilled. The method is acceptable for the quantification of methyleugenol in formulation Mevalone with a Limit of Quantification of 16.8 mg/kg.

5.2.1.3 Description of analytical methods for the determination of formulants (KCP 5.1.1)

This information is not required under Regulation (EC) No.1107/2009.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There is no CIPAC method available for the determination of eugenol, geraniol and thymol in plant protection products.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

All information and validation data were provided in the EU review of eugenol, geraniol and thymol compounds and were considered adequate within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU.

An overview on the acceptable methods and possible data gaps for analysis of residues of eugenol, geraniol and thymol for the generation of pre-authorization data is given in the following table. These studies have been submitted during the renewal of approval of the active substances eugenol, geraniol and thymol.

For the detailed evaluation of new studies it is referred to Appendix 2.

Table 5.2-2: Validated methods for the generation of pre-authorization data

Component of residue definition: eugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Grape fruit (Residues)	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Bailey A., 2007 Brown D., 2012 EU agreed in DAR (2011) under data point IIA 4.3/02
				Jones S., 2012 Submitted during the renewal of approval of eugenol
	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Bailey A., 2008 EU agreed in DAR (2011) under data point IIA 4.3/01
	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Cheshire A., 2008 Brown D., 2007 Submitted during the renewal of approval of eugenol
	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of eugenol Refer to the post-registration method

Component of residue definition: eugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Apples (Residues)	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of eugenol Refer to the post-registration method
Body fluids (plasma and urine) (Toxicology)	Primary	Intended to be 0.01 mg/L	GC-MS (three ions monitored)	Driss F., 2021e Submitted during the renewal of approval of eugenol Refer to the post-registration method
Body tissues (meat and liver) (Toxicology)	Primary	Intended to be 0.01 mg/kg	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of eugenol Refer to the post-registration method
Avian diet (Ecotoxicology)	Primary	96.9 µg/g	GC-FID	Martin K.H., Nixon W.B., 2007 XXXXX EU agreed in DAR (2011) under data point IIA 8.1.2/01
Water (Ecotoxicology)	Primary	3 mg/L	GC-MS	XXXX, 2008a EU agreed in DAR (2011) under data point IIA 8.2.1.1/01
	Primary	3 mg/L	GC-MS	XXXXX, 2008b EU agreed in DAR (2011) under data point IIA 8.2.1.2/01
	Primary	1 mg/L	GC-MS	Pavić B., Wydra V., 2008 EU agreed in DAR (2011) under data point IIA 8.3.1.1/01
	Primary	3.5 µg/L	GC-MS (three ions monitored)	Egeler P., 2021 Submitted during the renewal of approval of eugenol
	Primary	4 mg/L	GC-MS (three ions monitored)	Meister Werner A., Wydra V., 2008 EU agreed in DAR (2011) under data point IIA 8.4/01
	Primary	0.163 mg/L	GC-MS	XXXXXXX, 2008a EU agreed in DAR (2011) under data point IIIA 10.2.2.1/01
	Primary	0.163 mg/L	GC-MS	XXXXXXX, 2008b EU agreed in DAR (2011) under data point IIIA 10.2.2.2/01
	Primary	0.33 mg/L	GC-MS	XXXXXXX, 2008c EU agreed in DAR (2011) under data point IIIA 10.2.2.3/01
	Primary	100.6 mg/L	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019b Submitted during the renewal of approval of eugenol
Sugar feeding solution (Ecotoxicology)	Primary	20.28 mg/kg	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019a Submitted during the renewal of approval of eugenol
Water, buffer	Primary	2.110 mg/L in	HPLC-UV	Lingott J., 2020a

Component of residue definition: eugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
solutions,... (Properties)		aqueous solution 6.785 mg/L in n-octanol solution		Submitted during the renewal of approval of eugenol

Component of residue definition: geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Grape fruit (Residues)	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Bailey A., 2007 Brown D., 2012 EU agreed in DAR (2011) under data point IIA 4.3/02
				Jones S., 2012 Submitted during the renewal of approval of geraniol
	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Bailey A., 2008 Brown D., 2007 EU agreed in DAR (2011) under data point IIA 4.3/01
	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Cheshire A., 2008 Submitted during the renewal of approval of geraniol
Apples (Residues)	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of geraniol Refer to the post-registration method
				Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of geraniol Refer to the post-registration method

Component of residue definition: geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Tomato, cucumber and strawberry (Residues)	Primary	0.05 mg/kg	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of geraniol Refer to the post-registration method
Body fluids (Toxicology)	Primary	Intended to be 0.01 mg/L	GC-MS (three ions monitored)	Driss F., 2021e Submitted during the renewal of approval of geraniol Refer to the post-registration method
Body tissues (meat and liver) (Toxicology)	Primary	Intended to be 0.01 mg/kg	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of geraniol Refer to the post-registration method
Avian diet (Ecotoxicology)	Primary	196.8 µg/g	GC-FID	Martin K.H., Nixon W.B., 2007 XXXXXX, 2007 EU agreed in DAR (2011) under data point IIA 8.1.2/01
Water (Ecotoxicology)	Primary	2.0 mg/L	GC-MS	XXXXXXd EU agreed in DAR (2011) under data point IIA 8.2.1.1/01
	Primary	2.0 mg/L	GC-MS	XXXXXXe EU agreed in DAR (2011) under data point IIA 8.2.1.2/01
	Primary	3.0 mg/L	GC-MS	Grade R., Wydra V., 2007a EU agreed in DAR (2011) under data point IIA 8.3.1.1/01
	Primary	12.0 µg/L	GC-MS (three ions monitored)	Egeler P., 2021 Submitted during the renewal of approval of geraniol
	Primary	1.77 mg/L	GC-MS	XXXXXXf EU agreed in DAR (2011) under data point IIA 8.4/01
	Primary	0.03 mg/L	GC-MS (three ions monitored)	Seidel U., Emnet P., 2021 Submitted during the renewal of approval of geraniol
	Primary	0.98 mg/L	GC-MS	XXXXXXa EU agreed in DAR (2011) under data point IIIA 10.2.2.1/01
	Primary	1.31 mg/L	GC-MS	XXXXXXb EU agreed in DAR (2011) under data point IIIA 10.2.2.2/01
	Primary	0.66 mg/L	GC-MS	XXXXXXc EU agreed in DAR (2011) under data point IIIA 10.2.2.3/01
	Primary	196.5 mg/L	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019b Submitted during the renewal of approval of geraniol
Sugar feeding solution (Ecotoxicology)	Primary	40.9 mg/kg	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019a Submitted during the renewal of approval of geraniol

Component of residue definition: geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Water, buffer solutions,... (Properties)	Primary	0.2515 mg/L in aqueous solution 0.45 mg/L in n-octanol solution	HPLC-UV	Lingott J., 2020b Submitted during the renewal of approval of geraniol

Component of residue definition: thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Grape fruit (Residues)	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Bailey A., 2007 Brown D., 2012 EU agreed in DAR (2011) under data point IIA 4.3/02
				Jones S., 2012 Submitted during the renewal of approval of thymol
	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Bailey A., 2008 Brown D., 2007 EU agreed in DAR (2011) under data point IIA 4.3/01
	Primary	0.05 mg/kg	GC-MS (three ions monitored)	Cheshire A., 2008 Submitted during the renewal of approval of thymol
Apples (Residues)	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of thymol Refer to the post-registration method
	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of thymol Refer to the post-registration method

Component of residue definition: thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Tomato, cucumber and strawberry (Residues)	Primary	0.05 mg/kg	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of geraniol Refer to the post-registration method
Body fluids (Toxicology)	Primary	Intended to be 0.01 mg/L	GC-MS (three ions monitored)	Driss F., 2021e Submitted during the renewal of approval of thymol Refer to the post-registration method
	Primary	10 µg/L in mouse plasma 10 µg/L in rat plasma 20 mg/mL in olive oil and 1% methyl cellulose	GC-MS (three ions monitored)	Brice A., Heslop D., 2009 XXXXXX, 2009 XXXXXX, 2009 Submitted during the renewal of approval of thymol
Body tissues (meat and liver) (Toxicology)	Primary	Intended to be 0.01 mg/kg	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of thymol Refer to the post-registration method
Avian diet (Ecotoxicology)	Primary	194.2 µg/g	GC-FID	Martin K.H., Nixon W.B., 2007 XXXXXX, 2007 EU agreed in DAR (2011) under data point IIA 8.1.2/01
Water (Ecotoxicology)	Primary	1 mg/L	GC-MS	XXXXXXXg EU agreed in DAR (2011) under data point IIA 8.2.1.1/01
	Primary	2 mg/L	GC-MS	XXXXXXXh EU agreed in DAR (2011) under data point IIA 8.2.1.2/01
	Primary	1 mg/L	GC-MS	Grade R., Wydra V., 2007b EU agreed in DAR (2011) under data point IIA 8.3.1.1/01
	Primary	28 µg/L	GC-MS (three ions monitored)	Egeler P., 2021 Submitted during the renewal of approval of thymol
	Primary	3.2 mg/L	GC-MS	Grade R., Wydra V. Hoffmann K., Wydra V., 2011 EU agreed in DAR (2011) under data point IIA 8.4/01
	Primary	0.332 mg/L	GC-MS	XXXXXXXa EU agreed in DAR (2011) under data point IIIA 10.2.2.1/01
	Primary	1.33 mg/L	GC-MS	XXXXXXXb EU agreed in DAR (2011) under data point IIIA 10.2.2.2/01
	Primary	0.065 mg/L	GC-MS	XXXXXXXc EU agreed in DAR (2011) under data point IIIA 10.2.2.3/01
	Primary	199.9 mg/L	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019b

Component of residue definition: thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
				Submitted during the renewal of approval of thymol
Sugar feeding solution (Ecotoxicology)	Primary	41.58 mg/kg	HPLC-DAD	Aversa S., 2019 Pecorari F., 2019a Submitted during the renewal of approval of thymol
Water, buffer solutions,... (Properties)	Primary	0.1255 mg/L in aqueous solution 3.38 mg/L in n-octanol solution	HPLC-UV	Lingott J., 2020 Submitted during the renewal of approval of thymol

Component of residue definition: methyleugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Grape fruit (Residues)	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G, 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of eugenol Refer to the post-registration method
Apples (Residues)	Primary	0.01 mg/kg	GC-MS (three ions monitored)	Chadwick G, 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of eugenol Refer to the post-registration method
Body fluids (Toxicology)	Primary	Intended to be 0.01 mg/L	GC-MS (three ions monitored)	Driss F., 2021e Submitted during the renewal of approval of eugenol Refer to the post-registration method
	Primary	Intended to be 0.01 mg/kg	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of eugenol Refer to the post-registration method

5.3 Methods for post-authorization control and monitoring purposes (KCP 5.2)

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substances and relevant impurity in the plant protection product shall be submitted, unless the applicant shows that these methods already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

The applicant confirms that in its expert opinion, the methods submitted under point 5.2.1 can be applied for post-authorisation and monitoring and therefore additional methods under this point have not been submitted.

5.3.2 Description of analytical methods for the determination of residues of eugenol (KCP 5.2)

5.3.2.1 Overview of residue definitions and levels for which compliance is required

Table 5.3-1: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content	It is proposed that eugenol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Plant, high water content			
Plant, high protein/high starch content (dry commodities)			
Plant, high oil content			
Plant, difficult matrices (hops, spices, tea)			
Muscle	It is proposed that eugenol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	Eugenol and methyleugenol	0.05 mg/kg	SANTE/2020/12830, Rev.1; general limit
Drinking water (Human toxicology)	Eugenol and methyleugenol	0.1 µg/L	SANTE/2020/12830, Rev.1 general limit for drinking water
Surface water (Ecotoxicology)	Eugenol and methyleugenol	0.1 mg/L	NOEC value for <i>Daphnia</i>
Air	Not applicable as eugenol is listed on Annex IV to Regulation (EC) No 396/2005 of the European Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.		
Tissue (meat or liver)	Eugenol and methyleugenol	0.01 mg/kg	SANTE/2020/12830, Rev.1; general limit
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev.1; general limit

5.3.2.2 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of eugenol and its relevant impurity methyleugenol in plant matrices is given in the following tables.

Not applicable, it is proposed that eugenol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required for plant matrices.

However, analytical methods for quantification of eugenol and its relevant impurity methyleugenol in grapes and apples were developed.

These studies have been submitted during the renewal of approval of the active substance eugenol.

Table 5.3-2: Validated methods for food and feed of plant origin (required for all matrix types, “difficult” matrix only when indicated by intended GAP)

Component of residue definition: no definition of residue for eugenol					
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed	
High water content	Primary	0.01 mg/kg in apples for eugenol 0.01 mg/kg in apples for methyleugenol	GC-MS (three ions monitored)	Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of eugenol	
	ILV	Not required as no definition of residue or MRL has been set for the active substance eugenol.			
	Confirmatory (if required)	Not required			
High acid content	Primary	0.01 mg/kg in grapes for eugenol 0.01 mg/kg in grapes for methyleugenol	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of eugenol	
	ILV	Not required as no definition of residue or MRL has been set for the active substance eugenol.			
	Confirmatory (if required)	Not required			

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	No definition of residue or MRL has been set for the active substance eugenol in food and feed of plant origin.

5.3.2.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of eugenol and its relevant impurity methyleugenol in animal matrices is given in the following tables.

Not applicable, it is proposed that eugenol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition for animal matrices would not be required.

However, analytical methods for quantification of eugenol and its relevant impurity methyleugenol in body fluids and tissues were developed.

These studies have been submitted during the renewal of approval of the active substance eugenol.

Table 5.3-4: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: no definition of residue for eugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk, eggs, fat	Primary	Not required as no definition of residue or MRL has been set for the active substance eugenol.		
	ILV			
	Confirmatory (if required)			
Muscle (meat),	Primary	0.01 mg/kg in meat	GC-MS (three ions	Driss F., 2021f

Component of residue definition: no definition of residue for eugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
liver		for eugenol 0.01 mg/kg in liver for eugenol 0.01 mg/kg in meat for methyleugenol 0.01 mg/kg in liver for methyleugenol	monitored)	Submitted during the renewal of approval of eugenol
	ILV	Not required as no definition of residue or MRL has been set for the active substance eugenol.		
	Confirmatory (if required)	Not required		

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	No definition of residue or MRL has been set for the active substance eugenol in food and feed of animal origin.

5.3.2.4 Description of methods for the analysis of soil (KCP 5.2)

According to Guidance document SANCO/825/00 rev.8.1 and the subsequent guidance document SANTE/2020/12830 rev.1, analytical methods for residues in soil are not necessary if more than 90% of the start concentration of the active substance and its relevant metabolites are degraded within 3 days (DT90 < 3 days). This is the case for eugenol and methyleugenol.

5.3.2.5 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of eugenol and its relevant impurity methyleugenol in surface and drinking water is given in the following tables.

These studies have been submitted during the renewal of approval of the active substance eugenol.

Table 5.3-6: Validated methods for water (if appropriate)

Component of residue definition: eugenol and methyleugenol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water and surface water	Primary	0.1 µg/L for eugenol	LC-MS/MS (Method with C18 column)	Chambers J., 2020a Submitted during the renewal of approval of eugenol
	Primary	0.1 µg/L for methyleugenol	GC-MS (three ions monitored)	
	ILV	Study on-going and will be submitted as part of the renewal of approval of eugenol		
	Confirmatory	0.1 µg/L for eugenol	LC-MS/MS (Method with Pentafluorophenyl column)	Chambers J., 2020a Submitted during the renewal of approval of eugenol
		Not required for methyleugenol		

5.3.2.6 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of eugenol and its relevant impurity methyleugenol in air is given in the following tables.

Not applicable as eugenol is listed on Annex IV to Regulation (EC) No 396/2005 of the European

Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.

There is no definition of residue for eugenol.

However, analytical methods for quantification of eugenol and its relevant impurity methyleugenol in air were developed.

These studies have been submitted during the renewal of approval of the active substance eugenol.

Table 5.3-7: Validated methods for air (if appropriate)

Component of residue definition: no definition of residue for eugenol			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	1.2 µg/m ³ for eugenol 1.2 µg/m ³ for methyleugenol	GC-MS (three ions monitored)	Chambers J., 2020b Submitted during the renewal of approval of eugenol
Confirmatory	Not required		

5.3.2.7 Description of methods for the analysis of body fluids and tissues (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of eugenol and its relevant impurity methyleugenol in body fluids and tissues is given in the following table.

These studies have been submitted during the renewal of approval of the active substance eugenol.

Table 5.3-8: Methods for body fluids and tissues (if appropriate)

Component of residue definition: eugenol and methyleugenol			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/L in plasma for eugenol 0.01 mg/L in urine for eugenol	GC-MS (three ions monitored)	Driss F., 2021e Submitted during the renewal of approval of eugenol
	0.01 mg/L in plasma for methyleugenol 0.01 mg/L in urine for methyleugenol		
	0.01 mg/kg in meat for eugenol 0.01 mg/kg in liver for eugenol 0.01 mg/kg in meat for methyleugenol 0.01 mg/kg in liver for methyleugenol	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of eugenol
Confirmatory	Not required		

5.3.2.8 Other studies/ information

No other studies available

5.3.3 Description of analytical methods for the determination of residues of geraniol (KCP 5.2)

5.3.3.1 Overview of residue definitions and levels for which compliance is required

Table 5.3-9: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content	It is proposed that geraniol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Plant, high water content			
Plant, high protein/high starch content (dry commodities)			
Plant, high oil content			
Plant, difficult matrices (hops, spices, tea)			
Muscle	It is proposed that geraniol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	Geraniol	0.05 mg/kg	SANTE/2020/12830, Rev.1; general limit
Drinking water (Human toxicology)	Geraniol	0.1 µg/L	SANTE/2020/12830, Rev.1 general limit for drinking water
Surface water (Ecotoxicology)	Geraniol	0.1 mg/L	NOEC value for <i>Daphnia</i>
Air	Not applicable as geraniol is listed on Annex IV to Regulation (EC) No 396/2005 of the European Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.		
Tissue (meat or liver)	Geraniol	0.01 mg/kg	SANTE/2020/12830, Rev.1; general limit
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev.1; general limit

5.3.3.2 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of geraniol in plant matrices is given in the following tables.

Not applicable as it is proposed that geraniol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition for plant matrices would not be required.

However, analytical methods for quantification of geraniol in grapes and apples were developed.

These studies have been submitted during the renewal of approval of the active substance geraniol.

Table 5.3-10: Validated methods for food and feed of plant origin (required for all matrix types, “difficult” matrix only when indicated by intended GAP)

Component of residue definition: no definition of residue for geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg in apples	GC-MS (three ions monitored)	Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of geraniol
	Primary	0.05 mg/kg in cucumber and tomatoes	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of geraniol
	ILV	Not required as no definition of residue or MRL has been set for the active substance geraniol.		
	Confirmatory (if required)	Not required		
High acid content	Primary	0.01 mg/kg in grapes	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of geraniol
	Primary	0.05 mg/kg in strawberries	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of geraniol
	ILV	Not required as no definition of residue or MRL has been set for the active substance geraniol.		
	Confirmatory (if required)	Not required		

Table 5.3-11: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	No definition of residue or MRL has been set for the active substance geraniol in food and feed of plant origin.

5.3.3.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of geraniol in animal matrices is given in the following tables.

Not applicable, it is proposed that geraniol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition for animal matrices would not be required.

However, analytical methods for quantification of geraniol in body fluids and tissues were developed. These studies have been submitted during the renewal of approval of the active substance geraniol.

Table 5.3-12: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: no definition of residue for geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk, eggs, fat	Primary	Not required as no definition of residue or MRL has been set for the active substance geraniol.		
	ILV			
	Confirmatory (if required)			
Muscle (meat), liver	Primary	0.01 mg/kg in meat 0.01 mg/kg in liver	GC-MS (three ions monitored) for meat LC-MS/MS (two mass transitions) for liver	Driss F., 2021f Submitted during the renewal of approval of geraniol
	ILV	Not required as no definition of residue or MRL has been set for the active substance geraniol.		
	Confirmatory (if required)	Not required		

Table 5.3-13: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	No definition of residue or MRL has been set for the active substance geraniol in food and feed of animal origin.

5.3.3.4 Description of methods for the analysis of soil (KCP 5.2)

According to Guidance document SANCO/825/00 rev.8.1 and the subsequent guidance document SANTE/2020/12830 rev.1, analytical methods for residues in soil are not necessary if more than 90% of the start concentration of the active substance and its relevant metabolites are degraded within 3 days ($DT_{90} < 3$ days). This is the case for geraniol.

5.3.3.5 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of geraniol in surface and drinking water is given in the following tables.

These studies have been submitted during the renewal of approval of the active substance geraniol.

Table 5.3-14: Validated methods for water (if appropriate)

Component of residue definition: geraniol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water and surface water	Primary	0.1 µg/L	GC-MS (three ions monitored)	Chambers J., 2020a Submitted during the renewal of approval of geraniol
	ILV	Study on-going and will be submitted as part of the renewal of approval of geraniol		
	Confirmatory	Not required		

5.3.3.6 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of geraniol in air is given in the following tables.

Not applicable as geraniol is listed on Annex IV to Regulation (EC) No 396/2005 of the European Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.

There is no definition of residue for geraniol.

However, analytical methods for quantification of geraniol in air were developed.

These studies have been submitted during the renewal of approval of the active substance geraniol.

Table 5.3-15: Validated methods for air (if appropriate)

Component of residue definition: no definition of residue for geraniol			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	1.2 µg/m ³	GC-MS (three ions monitored)	Chambers J., 2020b Submitted during the renewal of approval of geraniol
Confirmatory	Not required		

5.3.3.7 Description of methods for the analysis of body fluids and tissues (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of geraniol in body fluids and tissues is given in the following table.

These studies have been submitted during the renewal of approval of the active substance geraniol.

Table 5.3-16: Methods for body fluids and tissues (if appropriate)

Component of residue definition: geraniol			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/L in plasma 0.01 mg/L in urine	GC-MS (two ions monitored) plus LC-MS/MS (one mass transition)	Driss F., 2021e Submitted during the renewal of approval of geraniol
	0.01 mg/kg in meat 0.01 mg/kg in liver	GC-MS (three ions monitored) for meat LC-MS/MS (two mass transitions) for liver	Driss F., 2021f Submitted during the renewal of approval of geraniol
Confirmatory	Not required		

5.3.3.8 Other studies/ information

No other studies available.

5.3.4 Description of analytical methods for the determination of residues of thymol (KCP 5.2)

5.3.4.1 Overview of residue definitions and levels for which compliance is required

Table 5.3-17: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content	It is proposed that thymol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Plant, high water content			
Plant, high protein/high starch content (dry commodities)			

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high oil content	It is proposed that thymol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition would not be required.		
Plant, difficult matrices (hops, spices, tea)			
Muscle			
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	Thymol	0.05 mg/kg	SANTE/2020/12830, Rev.1; general limit
Drinking water (Human toxicology)	Thymol	0.1 µg/L	SANTE/2020/12830, Rev.1 general limit for drinking water
Surface water (Ecotoxicology)	Thymol	0.1 mg/L	NOEC value for <i>Daphnia</i>
Air	Not applicable as thymol is listed on Annex IV to Regulation (EC) No 396/2005 of the European Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.		
Tissue (meat or liver)	Thymol	0.01 mg/kg	SANTE/2020/12830, Rev.1; general limit
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev.1; general limit

5.3.4.2 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of thymol in plant matrices is given in the following tables.

Not applicable, it is proposed that thymol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition for plant matrices would not be required.

However, analytical methods for quantification of thymol in grapes and apples were developed.

These studies have been submitted during the renewal of approval of the active substance thymol.

Table 5.3-18: Validated methods for food and feed of plant origin (required for all matrix types, “difficult” matrix only when indicated by intended GAP)

Component of residue definition: no definition of residue for thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg in apples	GC-MS (three ions monitored)	Chadwick G., 2021b Driss F., 2021c Driss F., 2021d Submitted during the renewal of approval of thymol
	Primary	0.05 mg/kg in cucumber and tomatoes	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of thymol
	ILV	Not required as no definition of residue or MRL has been set for the active substance thymol.		
	Confirmatory (if required)	Not required		

Component of residue definition: no definition of residue for thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High acid content	Primary	0.01 mg/kg in grapes	GC-MS (three ions monitored)	Chadwick G., 2021a Driss F., 2021a Driss F., 2021b Submitted during the renewal of approval of thymol
	Primary	0.05 mg/kg in strawberries	LC-MS/MS (two ion transitions monitored)	Wiesner F., Breyer N., 2017 Submitted during the renewal of approval of thymol
	ILV	Not required as no definition of residue or MRL has been set for the active substance thymol.		
	Confirmatory (if required)	Not required		

Table 5.3-19: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	No definition of residue or MRL has been set for the active substance thymol in food and feed of plant origin.

5.3.4.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of thymol in animal matrices is given in the following tables.

Not applicable, it is proposed that thymol is included into Annex IV to Regulation (EC) No. 396/2005 and so a residue definition for animal matrices would not be required.

However, analytical methods for quantification of thymol in body fluids and tissues were developed. These studies have been submitted during the renewal of approval of the active substance thymol.

Table 5.3-20: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: no definition of residue for thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk, eggs, fat	Primary	Not required as no definition of residue or MRL has been set for the active substance thymol.		
	ILV			
	Confirmatory (if required)			
Muscle (meat), liver	Primary	0.01 mg/kg in meat 0.01 mg/kg in liver	GC-MS (three ions monitored)	Driss F., 2021f Submitted during the renewal of approval of thymol
	ILV	Not required as no definition of residue or MRL has been set for the active substance thymol.		
	Confirmatory (if required)	Not required		

Table 5.3-21: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	No definition of residue or MRL has been set for the active substance

	Method for products of animal origin
	thymol in food and feed of animal origin.

5.3.4.4 Description of methods for the analysis of soil (KCP 5.2)

According to Guidance document SANCO/825/00 rev.8.1 and the subsequent guidance document SANTE/2020/12830 rev.1, analytical methods for residues in soil are not necessary if more than 90% of the start concentration of the active substance and its relevant metabolites are degraded within 3 days ($DT_{90} < 3$ days). This is the case for thymol.

5.3.4.5 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of thymol in surface and drinking water is given in the following tables.

These studies have been submitted during the renewal of approval of the active substance thymol.

Table 5.3-22: Validated methods for water (if appropriate)

Component of residue definition: thymol				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water and surface water	Primary	0.1 µg/L	GC-MS (three ions monitored)	Chambers J., 2020a Submitted during the renewal of approval of thymol
	ILV	Study on-going and will be submitted as part of the renewal of approval of thymol		
	Confirmatory	Not required		

5.3.4.6 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of thymol in air is given in the following tables.

Not applicable as thymol is listed on Annex IV to Regulation (EC) No 396/2005 of the European Parliament according to COMMISSION REGULATION (EU) 2015/896 of 11 June 2015.

There is no definition of residue for thymol.

However, analytical methods for quantification of thymol in air were developed.

These studies have been submitted during the renewal of approval of the active substance thymol.

Table 5.3-23: Validated methods for air (if appropriate)

Component of residue definition: no definition of residue for thymol in air			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	1.2 µg/m ³	GC-MS (three ions monitored)	Chambers J., 2020b Submitted during the renewal of approval of thymol
Confirmatory	Not required		

5.3.4.7 Description of methods for the analysis of body fluids and tissues (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of thymol in body fluids and tissues is given in the following table.

These studies have been submitted during the renewal of approval of the active substance thymol.

Table 5.3-24: Methods for body fluids and tissues (if appropriate)

Component of residue definition: thymol			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/L in plasma 0.01 mg/L in urine	GC-MS (two ions monitored) plus LC-MS/MS (one mass transition)	Driss F., 2021e Submitted during the renewal of approval of thymol
	0.01 mg/kg in meat 0.01 mg/kg in liver	GC-MS (three ions monitored) for meat GC-MS (two ions monitored) plus LC-MS/MS (one mass transition) for liver	Driss F., 2021f Submitted during the renewal of approval of thymol
Confirmatory	Not required		

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 2.

5.3.4.8 Other studies/ information

No other studies available.

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

These studies have also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1/02	Wronska, L.	2020a	Validation of Analytical Method TS20010-1 for the determination of Active Ingredient Content in Cedroz and Mevalone Formulations Battelle UK Ltd. Report No. TS/20/010/1 GLP Unpublished	N	Eden Research plc
KCP 5.1.1/06	Wronska, L.	2020b	Validation of an Analytical Method TS21001-1 for the determination of Impurity Methy-Eugenol in Mevalone Formulations Battelle UK Ltd. Report No. TS/21/001/1 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/18	Bates G.J.D.	2014	Ambient storage stability trial for the 3AEY formulation G.C. Laboratories Ltd Report No. J19053 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/03 KCA 4.1.2/03 (eugenol) KCA 4.1.2/03 (geraniol) KCA 4.1.2/05 (thymol)	Cheshire A.	2008	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern and Northern France, 2007 Agriseach UK Ltd Report No. AF/12268/ED GLP Unpublished	N	Eden Research plc
KCP 5.1.2/05 KCA 4.1.2/05 (eugenol/geraniol) KCA 4.1.2/08 (thymol)	Chadwick G.	2021a	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to grapevine, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 Eurofins Agroscience Services Report No. S20-06337 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/06	Chadwick G.	2021b	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone	N	Eden Research

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCA 4.1.2/06 (eugenol/ geraniol) KCA 4.1.2/09 (thymol)			(3AEY / EDN-004) to apple, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 Eurofins Agroscience Services Report No. S20-06361 GLP Unpublished		plc
KCA 4.1.2/21 (eugenol)	Egeler P	2021a	Eugenol : A Study on the Chronic Toxicity to <i>Daphnia magna</i> - Analysis of Test Solutions - ECT Oekotoxikologie GmbH Report No. 20GC3DB GLP Unpublished	N	Eden Research plc
KCA 4.1.2/42 (geraniol)	Schrag K.	2021	A Study on the Chronic Toxicity to <i>Daphnia magna</i> - Analysis of Test Solutions – CIP GmbH report n°20GC1DB GLP Unpublished	N	Eden Research plc
KCA 4.1.2/44 (geraniol)	Seidel U., Emnet P.	2021	Geraniol: Toxicity to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test CIP GmbH report No 155771210 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/21 (thymol)	Egeler P.	2021b	A Study on the Chronic Toxicity to <i>Daphnia magna</i> ECT GmbH Report No. 20GC2DB GLP Unpublished	N	Eden Research plc
KCP 5.1.2/12 KCA 4.1.2/23 (eugenol) KCA 4.1.2/45 (geraniol) KCA 4.1.2/23 (thymol)	Aversa S.	2019	Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document) BioTecnologie BT S.r.l. Report No. BT081/19 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/13	Pecorari F.	2019a	Chronic oral effects of ARAW on adult worker honeybees <i>Apis mellifera</i> L., 10-day feeding laboratory test BioTecnologie BT S.r.l. Report No. BT059/19 GLP	N	Eden Research plc

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCP 5.1.2/14	Pecorari F.	2019b	Effects of ARAW on honeybees (<i>Apis mellifera</i> L.) 22-day larval toxicity test with repeated exposure BioTecnologie BT S.r.l. Report No. BT060/19 GLP Unpublished	N	Eden Research plc
KCA 4.2/01 (geraniol/ thymol)	Wiesner F., Breyer N.	2017	Validation of an Analytical Method for the Determination of Residues of Geraniol and Thymol in Tomato, Cucumber and Strawberry Eurofins GmbH Report No. S16-03357 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/25 (eugenol)	Lingott J.	2020a	Partition coefficient of Eugenol (Shake-Flask Method) Eurofins GmbH Report No. S20-06643 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/47 (geraniol)	Lingott J.	2020b	Partition coefficient of Geraniol (Shake-Flask Method) Eurofins GmbH Report No. S20-06645 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/25 (thymol)	Lingott J.	2020	Partition coefficient of Thymol (Shake-Flask Method) Eurofins Agroscience Services EcoChem GmbH Report No. S20-06644 GLP Unpublished	N	Eden Research plc
KCP 5.2/01 KCA 4.2/01 (eugenol) KCA 4.2/02 (geraniol/ thymol)	Driss F	2021a	Validation of residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in grape Eurofins Agroscience Services Chem SAS Report No. S20-06528 GLP Unpublished	N	Eden Research plc
KCP 5.2/02 KCA 4.2/02 (eugenol) KCA 4.2/03 (geraniol/ thymol)	Driss F.	2021b	Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions Eurofins Agroscience Services Report No. S20-06526 GLP Unpublished	N	Eden Research plc

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2/05 KCA 4.2/05 (eugenol) KCA 4.2/06 (geraniol/ thymol)	Driss F	2021c	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in apple Eurofins Agroscience Services Chem SAS Report No. S20-06529 GLP Unpublished	N	Eden Research plc
KCP 5.2/06 KCA 4.2/06 (eugenol) KCA 4.2/07 (geraniol/ thymol)	Driss F	2021d	Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions Eurofins Agroscience Services Report No. S20-06527 GLP Unpublished	N	Eden Research plc
KCP 5.2/07 KCA 4.2/10 (eugenol) KCA 4.2/11 (geraniol/ thymol)	Driss F	2021e	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body fluid (plasma and urine) Eurofins Agroscience Services Chem SAS Report No. S20-06626 GLP Unpublished	N	Eden Research plc
KCP 5.2/08 KCA 4.2/11 (eugenol) KCA 4.2/12 (geraniol/ thymol)	Driss F	2021f	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body tissue (meat and liver) Eurofins Agroscience Services Chem SAS Report No. S20-06625 GLP Unpublished	N	Eden Research plc
KCP 5.2/09 KCA 4.2/07 (eugenol) KCA 4.2/08 (geraniol/ thymol)	Chambers J.	2020a	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Surface Water Report number TS/19/001 Battelle UK GLP Unpublished	N	Eden Research plc
KCP 5.2/10 KCA 4.2/09 (eugenol) KCA 4.2/10	Chambers J.	2020b	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Air – amended report Report number TS/19/003 Battelle UK GLP Unpublished	N	Eden Research plc

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
(geraniol/ thymol)					

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review

These studies have been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1/01	White G.A.	2007d	Validation of Analytical Method M619, Gas chromatographic determination of free and encapsulated thymol, eugenol and geraniol in formulations, for the 3AEY formulation G.C. Laboratories Ltd Report No. J16312 GLP Unpublished	N	Eden Research plc
KCP 5.1.1/03	Kant A.	2008	Terpene Release from Encapsulated Formulation 3AEY, Appendix 4 - RFM039_ExtrVal: Determination of free and encapsulated terpenes in formulation 3AEY: Validation of extraction and GC-MS methods Flavometrix Report No. RFM039_MainPhase Not GLP Unpublished	N	Eden Research plc
KCP 5.1.1/04	Kant A.	2008	Terpene Release from Encapsulated Formulation 3AEY, Appendix 5 - RFM039_APcIMSval: Validation of the APcI-MS Headspace Analysis Method Flavometrix Report No. RFM039_MainPhase Not GLP Unpublished	N	Eden Research plc
KCP 5.1.1/05	Bates G.J.D.	2012a	Validation of Analytical Method M737, Gas chromatographic determination of methyl eugenol in formulations, for low levels of methyl eugenol in the 3AEY formulation G.C. Laboratories Ltd Report No. J19055 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/01 (thymol)	Brice A., Heslop D.	2009	Validation for the Determination of Thymol in Dietary Formulation, Mouse Plasma and Rat Plasma Covance Laboratories Ltd Report No. 8201847-D2149 GLP	N	Eden Research plc

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCA 4.1.2/02 (thymol)	XXXXXX	2009	Thymol: Oral (Gavage) Administration Pharmacokinetic Study in the Mouse Covance Laboratories Ltd Report No. 8202028 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/03 (thymol)	XXXXXX	2009	Induction of chromosome aberrations in the bone marrow of treated rats Covance Laboratories Ltd Report No. 8201846 GLP Unpublished	Y	Eden Research plc
KCP 5.1.2/01 KCA 4.1.2/01 (eugenol/ geraniol) KCA 4.1.2/04 (thymol)	Bailey A.	2007	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern Europe (2006 – 2007) Agriseach UK Ltd Report No. AF/10728/ED GLP Unpublished	N	Eden Research plc
KCP 5.1.2/02 KCA 4.1.2/02 (eugenol) KCA 4.1.2/03 (geraniol) KCA 4.1.2/06 (thymol)	Bailey A.	2008	To determine the magnitude of geraniol, eugenol and thymol residues on the surface of grapes by deposit analysis resulting from sequential applications of 3AEY, in Southern Europe (2006) Agriseach UK Ltd Report No. AF/11125/ED GLP Unpublished	N	Eden Research plc
KCP 5.1.2/04 KCA 4.1.2/04 (eugenol/ geraniol) KCA 4.1.2/07 (thymol)	Jones S.	2012	Determination of natural background level residues of thymol, eugenol and geraniol in grapes Eurofins Agrosience Services Report No. S11-03787 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/07	Martin K.H., Nixon W.B.	2007	Analytical method verification for the determination of 3 AEY (thymol/geraniol/eugenol mixture) in avian diet Wildlife International Ltd Report No. 648C-101 GLP Unpublished	N	Eden Research plc

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2/08	XXXXXX	2007	3AEY (Thymol/Geraniol/Eugenol Mixture): a dietary LC50 study with the northern bobwhite Wildlife International Ltd Report No. 648-102 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/18 (eugenol)	XXXX	2008a	Acute Toxicity of EUGENOL to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 37984230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/19 (eugenol)	XXXXXXX	2008b	Acute Toxicity of EUGENOL to Zebra Fish (<i>Danio rerio</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 37983230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/20 (eugenol)	Pavié B., Wydra V.	2008	Acute Toxicity of EUGENOL to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test Ibacon GmbH Report No. 37982220 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/22 (eugenol)	Meister Werner A., Wydra V.	2008	Toxicity of EUGENOL to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test Ibacon GmbH Report No. 37981210 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/39 (geraniol)	XXXXXXX	2008d	Acute Toxicity of GERANIOL to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 34291230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/40 (geraniol)	XXXXXXX	2008e	Acute Toxicity of GERANIOL to Zebra Fish (<i>Danio rerio</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 34292230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/41 (geraniol)	Grade R., Wydra V.	2007a	Acute Toxicity of GERANIOL to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test Ibacon GmbH Report No. 34293220 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/43	Grade R., Wydra V.	2008f	Acute toxicity of GERANIOL to <i>Pseudokirchneriella subcapitata</i> in an Algal growth Inhibition Test	N	Eden Research

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
(geraniol)			Ibacon GmbH Report No. 34294210 GLP Unpublished		plc
KCA 4.1.2/18 (thymol)	XXXXXXX	2008g	Acute Toxicity of THYMOL CRYSTALS to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 34281230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/19 (thymol)	XXXXXXX	2008h	Acute Toxicity of THYMOL CRYSTALS to Zebra Fish (<i>Danio rerio</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 34282230 GLP Unpublished	Y	Eden Research plc
KCA 4.1.2/20 (thymol)	Grade R., Wydra V.	2007b	Acute Toxicity of THYMOL CRYSTALS to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test Ibacon GmbH Report No. 34283220 GLP Unpublished	N	Eden Research plc
KCA 4.1.2/22 (thymol)	Grade R., Wydra V. (Hoffmann K, Wydra V. amended report)	2008c, 2011	Toxicity of THYMOL CRYSTALS to <i>Pseudokirchneriella subcapitata</i> in an Algal growth Inhibition Test Ibacon GmbH Report No. 34284210 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/09	XXXXXXX	2008a	Acute Toxicity of 3AEY to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test Ibacon GmbH Report No. 34301230 GLP Unpublished	Y	Eden Research plc
KCP 5.1.2/10	Grade R., Wydra V.	2008b	Acute Toxicity of 3AEY to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test Ibacon GmbH Report No. 34302220 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/11	Grade R., Wydra V.	2008c	Toxicity of 3AEY to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test Ibacon GmbH Report No. 34303210 GLP Unpublished	N	Eden Research plc
KCP 5.1.2/15	White G.A.	2011	3AEY Formulation storage stability trial and physical / chemical tests	N	Eden Research

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Final report (24 months storage) G.C. Laboratories Ltd Report No. J16313 GLP Unpublished		plc
KCP 5.1.2/16	White G.A.	2007c	3AEY Formulation accelerated and cold storage temperature storage stability trials and physical chemical tests G.C. Laboratories Ltd Report No. J16537 GLP Unpublished	N	Eden Research plc
KCP 5.2/03 KCA 4.2/03 (eugenol) KCA 4.2/04 (geraniol/ thymol)	Brown D	2007	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. - 18° for 0, 1, 3, and 6 months (2006-2007) Eurofins Agrosience Services Report No. AD/11145/ED GLP Unpublished	N	Eden Research plc
KCP 5.2/04 KCA 4.2/04 (eugenol) KCA 4.2/05 (geraniol/ thymol)	Brown D	2012	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. - 18° for 0, 1, 3, 7, 14 and 28 days, 3, 6 and 12 months after treatment with 3AEY (6.4% w/w geraniol, 3.2% w/w eugenol and 6.4% w/w thymol) Eurofins Agrosience Services Report No. AD/12351/ED GLP Unpublished	N	Eden Research plc

List of data submitted by the applicant and not relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
-	-	-	-	-	-

List of data relied on and not submitted by the applicant but necessary for evaluation

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
-	-	-	-	-	-

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for eugenol

A 2.1.1 Methods used for the generation of pre-authorization data (KCP 5.1)

GRAPES (Residues)

KCP 5.1.2/01 (A 2.1.1/01 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/01 (A 2.1.1/01 of this dRR)
Report author	Bailey A.
Report year	2007
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern Europe (2006 – 2007)
Report No	AF/10728/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev 4
Deviations from current test guideline	The calibration range does not extend to 30% of the LOQ; Although three ions were monitored, except for specificity, validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with acetone. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and eugenol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°30422
Active substance content:	99.0%
Expiry date of lot/batch:	01 May 2009
Storage conditions:	stored at 4°C

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25 m x 0.32 mm internal diameter fused silica capillary coated with PAS-1701 (0.25 µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode,
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Eugenol: Approx. 8.0 min
Monitored ions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and acetone (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.01 and 0.1 mg/kg, extract concentrations are 0.005 µg/mL and 0.05 µg/mL respectively. For samples fortified at 0.05 and 0.2 mg/kg, extract concentrations are 0.025 µg/mL and 0.1 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for eugenol. Eight calibration standards were quantified. The equation to the calibration line was $7863559x - 113159$ and the correlation coefficient $R = 0.9987$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol.

No residues at the retention time of eugenol were found in untreated specimens taken from the control plots of each trial and subsequently used for procedural recovery tests.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol for each ion monitored. Full mass spectrometry scan of eugenol under the described analytical conditions was performed to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.05 mg/kg of eugenol residues in sample. The LOD was not stated.

Accuracy, Repeatability (precision):

Originally, it was expected that a limit of quantification of 0.01 mg/kg could be achieved therefore procedural recoveries were performed at levels of 0.01 and 0.1 mg/kg with each analytical batch. However due to problems associated with background interference and the inability to use three ions for quantification and qualification, the original target LOQ of 0.01 mg/kg could not be met. Following analysis of residue specimens, additional method validation, according to SANC0/3029/99 rev 4 (11/07/00), was performed by fortification of untreated grape specimens at 0.05 and 0.2 mg/kg with five replicates being analysed at each level. A composite untreated specimen from this study was prepared for this purpose. The analytical method was successfully validated at 0.05, 0.1 0.2 mg/kg.

Fortification level (mg/kg)	Mean recovery (%)	% RSD	n
Validation			
0.05	88	7.3	5
0.2	74	9.0	5
Combined	81	11.6	10
Procedural recoveries			
0.1	87	5.7	4

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability in the matrix was demonstrated by acceptable recoveries at 0.1 mg/kg fortification level.

Conclusions

Eugenol residues are extracted from raw commodity grapes sample with acetone, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

This analytical method for the determination of eugenol residues content in raw commodity grape samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. Although attempts to validate the method down to 0.01 mg/kg failed, the Limit of Quantification was validated at 0.05 mg/kg for eugenol residues in raw commodity grape samples. A full mass spectrometry scan of eugenol under the described analytical conditions is presented as part of the study to confirm analyte identification.

The method is acceptable for the quantification of eugenol residues in raw commodity grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.

The method is acceptable for the quantification of eugenol residues in grapes.

KCP 5.1.2/02 (A 2.1.1/02 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/02 (A 2.1.1/02 of this dRR)
Report author	Bailey A.
Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues on the surface of grapes by deposit analysis resulting from sequential applications of 3AEY, in Southern Europe (2006)
Report No	AF/11125/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC)
Deviations from current test guideline	Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°30422
Active substance content:	99.0%
Expiry date of lot/batch:	01 May 2009
Storage conditions:	stored at 4°C

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25m x 0.32 mm internal diameter fused silica capillary coated with PAS-1701 (0.25 µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode,
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Eugenol: Approx. 6.0 min
Monitored ions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

Extraction: A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.01, 0.1 and 1.0 mg/kg, extract concentrations are 0.01, 0.10 and 1.0 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen. Procedural recoveries were performed at 0.01 and 0.26 mg/kg, equivalent to 0.01 and 0.26 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for eugenol. Eight calibration standards were quantified. The equation to the calibration line was $2747635x - 28979$ and the correlation coefficient $R = 0.9990$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol.

No residues at the retention time of eugenol were found in untreated specimens taken from the control plots of each trial and subsequently used for procedural recovery tests.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol for each ion monitored. Full mass spectrometry scan of eugenol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.01 mg/kg of eugenol residue in sample.

The LOD was not stated.

Accuracy, Repeatability (precision):

Fortification level (mg/kg)	Mean recovery (%)	% RSD	n
Validation			
0.01	86	5.6	5
0.5	77	6.6	5
Combined	82	8.0	10
Procedural recoveries			
0.01	85	-	-
0.26	88	-	-
Mean	87	-	-

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability in the matrix was not investigated in this study.

Conclusions

Eugenol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS (three ions monitored).

This analytical method for the determination of eugenol residues content in grape samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. Full mass spectrometry scan of eugenol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification. The Limit of Quantification was 0.01 mg/kg for eugenol residues in grape samples.

The method presented herewith is satisfactory and can be applied to quantify eugenol residues in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level. It was performed under GLP, followed Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.

The method is acceptable for the quantification of eugenol residues in grapes.

KCP 5.1.2/03 (A 2.1.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The validation of the method for analysis of geraniol, eugenol and thymol residues in grapes was not previously evaluated at EU level. The method has been validated for the determination of geraniol, eugenol and thymol residues in grapes according to SANCO/3029/99 rev.4. Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD). The limit of quantification (LOQ) of the analytical method was 0.05 mg/kg of geraniol, eugenol and thymol residues in sample. The study has been accepted for the quantification of geraniol, eugenol and residue in grapes.
-------------------	--

Data point:	CP 5.1.2/03 (A 2.1.1/03 of this dRR)
Report author	Cheshire A.
Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern and Northern France, 2007
Report No	AF/12268/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) ENV/JM/MONO(2007)17
Deviations from current test guideline	Although three ions were monitored, validation data is available on one ion only.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, filtered over a 0.45 μm PTFE filter and eugenol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test Standards

Name: Eugenol
CAS number: 97-53-0

Source and lot/batch no.: Dr. Ehrenstorfer, lot n°30422
Active substance content: 99.0%
Expiry date of lot/batch: 01 May 2009
Storage conditions: stored at 4°C

Analysis parameters

Method type: GC-MS
Instrument: Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column: 30 m x 0.25 mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25 µm film)
Oven: 50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature: Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature: 275°C
Injection volume: Not stated
Injection mode: Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas: Helium
Flow rate: 1 mL/min
Ionization mode: Selected ion monitoring (SIM)
Retention time: Eugenol: Approx. 17.9 min
Monitored ions/transitions: 164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed.

For samples fortified at 0.05 mg/kg, 0.1 mg/kg, 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg, extract concentrations are 0.025 µg/mL, 0.05 µg/mL, 0.125 µg/mL, 0.25 µg/mL and 0.5 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in ethyl acetate to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.0125 – 1.0 µg/mL for eugenol. Seven calibration standards were quantified. The equation to the calibration line was $4522666x - 26511$ and the correlation coefficient $R = 0.9994$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.05 mg/kg of eugenol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL.

The LOD was not stated.

Accuracy, Repeatability (precision):

Accuracy was verified at five levels of fortification: 0.05, 0.1, 0.25, 0.5 and 1.0 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Eugenol recovery (%)
Procedural recovery	
0.05	101
0.25	105
0.05	108
0.5	96
0.05	102
0.1	98
0.05	100
0.25	98
0.05	99
1.0	98
Mean	101
%RSD	3.6 (n = 10)

In addition, accuracy and precision were also verified at two levels through five independent sample fortifications at 0.05 and 1.0 mg/kg. Analysis of untreated control samples indicated minimal level of interference from co-extracted material with residues ranging from 0.0113 to 0.0132 mg/kg. Eugenol residues were <0.05 mg/kg, however the mean content determined in the fortified samples were corrected for the mean determined in the untreated specimen.

Fortification level (mg/kg)	Eugenol recovery (%)	%RSD (n)
Validation		
0.05	101	4.3 (n = 5)
1.0	104	0.4 (n = 5)
Overall	102	3.3 (n = 10)

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability of eugenol residues in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Eugenol residues are extracted from raw commodity grapes sample with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

This analytical method for the determination of eugenol residues content in raw commodity grape samples has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled.

The method is acceptable for the quantification of eugenol residues in raw commodity grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was not previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.

The method is acceptable for the quantification of eugenol residues in grapes.

KCP 5.1.2/04 (A 2.1.1/04 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/04 (A 2.1.1/04 of this dRR)
Report author	Jones S.
Report year	2012
Report title	Determination of natural background level residues of thymol, eugenol and geraniol in grapes
Report No	S11-03787
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 EU 1999: 1607/VI/97 7032/VI/95 rev.5
Deviations from current test guideline	The method presented is in agreement with Guidance Documents SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4
Previous evaluation	Yes, evaluated and accepted in DAR (Addendum 2012) under data point IIA 6.3; IIIA 8.2
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and eugenol residues are quantified by GC-MS.

The method described herewith follows the validated EAS (formally Agrisearch) method 'Thymol, Eugenol & Geraniol/Crops/DB/08/1'. The method was fully validated in EAS study AF/10728/ED and EAS study AF/12268/ED, which are included in this submission.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Penta Manufacturing Co., lot n°112998
Active substance content:	99.4%
Expiry date of lot/batch:	15 March 2014
Storage conditions:	Refrigerated

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	30 m x 0.25 mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25 µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature:	Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature:	275°C
Injection volume:	Not stated
Injection mode:	Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Eugenol: Approx. 16.6 min
Monitored ions/transitions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed.

For samples fortified at 0.05, 0.1 and 0.2 mg/kg, extract concentrations are 0.025, 0.05 and 0.1 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in ethyl acetate to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

Linearity was investigated over the range 0.0125 – 1.0 µg/mL. Seven calibration standards were quantified. The equation to the calibration line was $4152952 \times - 29885$ and the correlation coefficient $R = 0.9998$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with eugenol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with eugenol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest for which acceptable recovery was demonstrated. LOQ is 0.05 mg/kg of eugenol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL.

Although the LOD was not stated, the lowest calibration standard concentration (0.0125 µg/mL), equivalent to a theoretical fortification level of 0.025 mg/kg is proposed as LOD.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels of fortification: 0.05, 0.1 and 0.2 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Eugenol recovery (%)
0.05	94
0.2	94
0.05	89
0.1	106
Mean	96
%RSD	7.5

Stability:

Stability of eugenol residues in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Eugenol residues are extracted from raw commodity grapes sample with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. The Limit of Quantification was 0.05 mg/kg for eugenol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of eugenol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission.

The method is acceptable for the quantification of eugenol residues in grapes.

KCP 5.2/03 (A 2.1.1/05 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.2/03 (A 2.1.1/05 of this dRR)
Report author	Brown D.
Report year	2007
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, and 6 months (2006-2007)
Report No	AD/11145/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev.4 SANCO/825/00 rev.7 EU Working Document 7032/VI/95 rev.5
Deviations from current test guideline	The calibration range does not extend to 50% of the LOQ; Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 6.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

The method was validated prior to use and validation data is presented in Agrisearch study AF/11125/ED

in the same test facility, which is included in this submission.

Materials and methods

Test material

Test Standards

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°30422
Active substance content:	99.0%
Expiry date of lot/batch:	01 May 2009
Storage conditions:	stored at 4°C

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25m x 0.32mm internal diameter fused silica capillary coated with PAS-1701 (0.25µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Eugenol: Approx. 13 min
Monitored ions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

Extraction: A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.1 mg/kg, extract concentration is 0.10 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Procedural recoveries were performed at 0.10 mg/kg, equivalent to 0.10 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for eugenol. Eight calibration standards were quantified. The equation to the calibration line was $6840946x - 160819$ and the correlation coefficient $R = 0.9975$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol.

Interferences or contamination peak were not observed.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape samples, treated grape samples, untreated grape samples spiked with eugenol for each ion monitored. Full mass spectrometry scan of eugenol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated in study number AF/11125/ED (Bailey, 2008). LOQ is 0.01 mg/kg of eugenol residue in sample. This level equates to a calibration standard concentration of 0.01 µg/ml.

Accuracy, Repeatability (precision):

Accuracy was verified at one level of fortification through procedural recoveries.

Fortification level (mg/kg)	0.1
Eugenol recovery (%)	86
% RSD	10.8
n	8

Stability:

Stability in the matrix was investigated at 1 month, 3 months and 6 months (34, 93 and 185 days).

Results were as follows:

Time point (days)	0	34	95	185
Recovery (%)	73 (n = 3)	15 (n = 3)	15 (n = 3)	17 (n = 3)

Eugenol is not stable for 1 month (34 days) in the matrix.

Conclusions

Eugenol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission. Full mass spectrometry scan of eugenol under the described analytical conditions was

performed as part of study AF/10728/ED to confirm analyte identification. The Limit of Quantification was 0.01 mg/kg for eugenol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of eugenol residues in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) and SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission.

The method is acceptable for the quantification of eugenol residues in grapes.

KCP 5.2/04 (A 2.1.1/06 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.2/04 (A 2.1.1/06 of this dRR)
Report author	Brown D.
Report year	2012
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, 7, 14 and 28 days, 3, 6 and 12 months after treatment with 3AEY (6.4% w/w geraniol, 3.2% w/w eugenol and 6.4% w/w thymol)
Report No	AD/12351/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) 7032/VI/95 rev.5 SANCO/3029/99 SANCO/825/00
Deviations from current test guideline	The method presented is in line with Guidance Document SANCO/825/00 rev.8.1
Previous evaluation	Partially evaluated (up to 28 days) and accepted in DAR (2011) under data point IIA 6.1.1/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with acetone. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and eugenol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

The method was validated prior to use and validation data is presented in Agrisearch study AF/10728/ED

in the same test facility, which is included in this submission. However due to unavailability of the chromatographic column, a new chromatographic column and oven conditions were used. These changes are not expected to have impact on the analytical method performance except for the retention time of the analytes.

Materials and methods

Test material

Test Standards

Name:	Eugenol
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°30422
Active substance content:	99.0%
Expiry date of lot/batch:	01 May 2009
Storage conditions:	stored at 4°C

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	2B-Multiresidue-1, 30m x 0.25mm id (0.25µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 40°C/min – 330°C (10 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode, temperature 250°C
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Approx. 11.5 min
Monitored ions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and acetone (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 1.0 mg/kg, extract concentration is 0.5 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

Specimen fortification was carried out using a 3AEY prepared in water.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for eugenol. Five calibration standards were quantified. The equation to the calibration line was $1931093x - 86320$ and the correlation coefficient $R = 0.9981$ (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is validated for this range of concentration.

Specificity:

Analysis of untreated control samples indicated minimal interference from co-extracted material with all untreated samples being <0.05 mg/kg.

Copies of relevant chromatograms are provided for standards of eugenol, untreated grape samples, treated grape samples, untreated and treated grape samples spiked with eugenol for each ion monitored. Full mass spectrometry scan of eugenol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated in study number AF/10728/ED (Bailey, 2007). LOQ is 0.05 mg/kg of eugenol residue in sample. This level equates to a calibration standard concentration of 0.025 µg/ml.

The LOD was not stated.

Accuracy, Repeatability (precision):

Accuracy was verified at one level of fortification through procedural recoveries at 0.5 mg/kg fortification.

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	92	81	88	82	86	104	90	107	70
Overall	Mean = 89%, RSD = 13.0%; n = 18								

Stability:

Stability of the eugenol residues in the matrix was investigated at 0, 1, 3, 7, 14, 27, 94, 189 and 366 days).

Results were as follows:

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	78	80	79	86	90	87	98	94	71
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)

Eugenol is stable for up to one year in the matrix when stored frozen at -18°C.

Conclusions

Eugenol residues are extracted from raw commodity grapes sample with acetone, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated for specificity, linearity, accuracy and precision of the method as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. The Limit of Quantification was 0.05 mg/kg for eugenol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of eugenol residues in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission.

The method is acceptable for the quantification of eugenol residues in grapes.

Avian diet (Ecotoxicology)

KCP 5.1.2/07 (A 2.1.1/07 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in avian diet was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/07 (A 2.1.1/07 of this dRR)
Report author	Martin K.H, Nixon W.B.
Report year	2007
Report title	Analytical method verification for the determination of 3 AEY (thymol/geraniol/eugenol mixture) in avian diet
Report No	648C-101
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and eugenol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test item

Name:

3AEY

Source and lot/batch no.:

Eden Research plc, lot n°YP0A3Y424-1.1

Active substance content:

Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg)
Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg)
Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)

Expiry date of lot/batch:

December 2008

Storage conditions: Ambient temperature

Analytical standard

Name: Eugenol (reference material)
CAS number: 97-53-0
Source and lot/batch no.: Sigma-Aldrich, lot n°3071X
Active substance content: 99.5% w/w (equivalent to 995 g/kg)
Expiry date of lot/batch: March 2010
Storage conditions: Ambient temperature

Analysis parameters

Method type: GC-FID
Instrument: Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column: DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven: 70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume: 2 µL
Injector temperature: 250°C
Injection mode: Splitless mode
Carrier gas: Helium, CHP ≈ 28 PSI
Flow rate: Hydrogen: 40 mL/min
Air: 450 mL/min
Retention time: Eugenol: Approx. 8.0 min

Sample preparation

10 g of fortified diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) to fit within the calibration range:

3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 9.69 µg eugenol¹/mL

20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL) equivalent to 6.46 µg eugenol¹/mL

Calibration standards

A eugenol stock solution was prepared by weighing 0.0503 g eugenol analytical standard in a 50 mL volumetric flask and brought to volume with acetone. The concentration of the stock solution was 1000 µg/mL. This stock solution was used to prepare a combined secondary standard solution containing all three substances (eugenol, geraniol and thymol), although only eugenol is considered in this summary. The secondary stock solution was prepared by transferring 10 mL of the primary stock solution into a 100 mL volumetric flask and adjusting to volume using 50:50 (v/v) acetone:hexane. The secondary stock concentration was 100 µg/mL. From this secondary stock solution, calibration standards spanning the concentration range 5.0 to 25.0 µg/mL in acetone/hexane 50:50 (v/v).

Accuracy and Recovery samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 96.9 ppm diet of eugenol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 710.6 ppm diet of eugenol)

Calculations

The concentration of eugenol found at the instrument was determined using the following equation:

$$\text{Eugenol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

¹ Based on a eugenol content of 3.23% w/w

$$\text{Eugenol (ppm)} = \left(\frac{\text{Eugenol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity}$$

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 µg/mL. Five calibration standards were quantified. The equation to the calibration line was 26.651x - 2.0061 and the correlation coefficient R = 0.9999 (y = peak area of eugenol, x = concentration of eugenol (in µg/mL)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, reagent blank samples, matrix blank samples, and matrix samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) was evaluated at the lowest analytical standard analyzed, and was observed to yield a detector response at least five times greater than the peak-to-peak background noise in the matrix blank extracts at the same dilution factor as the lowest matrix fortification.

ppm eugenol equivalent

$$= \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}}$$

The Limit of Quantification (LOQ) was set at 3000 ppm of test item based upon the lowest matrix fortification level analyzed concurrently with the samples, corresponding to 96.9 ppm (96.9 µg/g of diet).

Accuracy, Repeatability (precision):

Diet concentration (ppm)	Eugenol concentration in diet (ppm)	Measured Eugenol in diet (ppm)	% nominal	Mean (%)	SD	% RSD
3000	96.9	106	109.4	101.6%	7.0	6.9%
		95.8	98.9			
		90.2	93.1			
		105	108.4			
		95.1	98.1			
22000	710.6	626	88.1	99.7%	8.2	8.2%
		754	106.1			
		695	97.8			
		773	108.8			
		693	97.5			

The data indicates that the accuracy and precision of the method is acceptable at a level 96.9 ppm and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set 96.9 ppm (96.9 µg/g of diet).

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and eugenol is quantified by GC-FID. The method described was acceptably validated at concentration levels relevant to the test results. This analytical method for the determination of eugenol content in avian diet has been acceptably validated by definition of specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 96.9 µg eugenol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in avian diet.

KCP 5.1.2/08 (A 2.1.1/08 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in avian diet was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/08 (A 2.1.1/08 of this dRR)
Report author	XXXXXX
Report year	2007
Report title	3AEY (Thymol/Geraniol/Eugenol Mixture): a dietary LC50 study with the northern bobwhite
Report No	648-102
Document No	Not applicable
Guidelines followed in study	OECD 205 EPA OPPTS 850.2200
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and eugenol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission.

Materials and methods

Test material

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Eugenol (reference material)
-------	------------------------------

CAS number: 97-53-0
Source and lot/batch no.: Sigma-Aldrich, lot n°3071X
Active substance content: 99.5% w/w (equivalent to 995 g/kg)
Expiry date of lot/batch: March 2010
Storage conditions: Ambient temperature

Analysis parameters

Method type: GC-FID
Instrument: Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column: DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven: 70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume: 2 µL
Injector temperature: 250°C
Injection mode: Splitless mode
Carrier gas: Helium, CHP ≈ 28 PSI
Flow rate: Hydrogen: 40 mL/min
Air: 450 mL/min
Retention time: Eugenol: Approx. 8.07 min

Sample preparation

Samples of the test diets were collected to verify the test concentrations administered and to confirm the stability and homogeneity of the test substance in the diets.

10 g of diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) depending on their nominal concentration:

- 3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 9.69 µg eugenol²/mL
- 5000 ppm: 1/2 (extract concentration: 0.25 mg 3AEY/mL), equivalent to 8.075 µg eugenol⁸/mL
- 10 000 ppm: 1/5 (extract concentration: 0.20 mg 3AEY/mL) equivalent to 6.46 µg eugenol⁸/mL
- 20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL) equivalent to 6.46 µg eugenol⁸/mL

Calibration standards

Calibration standards in the range 5 – 25 µg/mL are prepared in acetone/hexane 50:50 (v:v).

Recovery and precision samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 96.9 ppm diet of eugenol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 710.6 ppm diet of eugenol)

Diet homogeneity

Homogeneity of the test substance in the diet was evaluated by collecting six samples from the 5000 and 20000 ppm test diets at the time of preparation on Day 0 and Day 1. Homogeneity samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. The homogeneity samples also served as verification samples for those concentrations.

Calculations

The concentration of eugenol found at the instrument was determined using the following equation:

² Based on a eugenol content of 3.23% w/w

$$\text{Eugenol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

$$\text{Eugenol (ppm)} = \left(\frac{\text{Eugenol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity}$$

$$\text{ppm eugenol equivalent} = \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}}$$

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 $\mu\text{g/mL}$. Five calibration standards were quantified. The equation to the calibration line was $27.1213x + 4.63823$ and the correlation coefficient $R = 0.9998$ (y = peak area of eugenol, x = concentration of eugenol (in $\mu\text{g/mL}$)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, matrix blank samples, and matrix samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) was set based upon the injection volume (2.00 μL) and the lowest standard concentration 5.00 $\mu\text{g a.i./mL}$. The LOD was set at 10.0 ng on-column.

The Limit of Quantification (LOQ) was set at 3000 ppm based upon the lowest matrix fortification level analyzed concurrently with the samples and the LOQ validated during the study 648-101C in the same test facility. The 5.00 $\mu\text{g a.i./mL}$ eugenol standard was equivalent to a calculated value of 50 ppm a.i. in the matrix blank extract. Measured values greater than or equal to the ppm a.i. equivalent were reported for each analyte.

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 3000 ppm (96.9 ppm diet of eugenol) and 22 000 ppm (710.6 ppm diet of eugenol).

Fortification level (mg/L)	3000 ppm	22 000 ppm	Mean (%)	%RSD	n
Accuracy Day 1	88%	83%	86	-	2
Accuracy Day 2	93%	87%	90	-	2
Overall			87.8	4.7	4

The results obtained confirmed the accuracy and the precision of the method at 3000 ppm (96.9 ppm diet of eugenol) and above.

Homogeneity:

Diet concentration (ppm)	Eugenol concentration in diet (ppm)		Measured Eugenol in diet (ppm)	% nominal	Mean (%)	SD	%RSD
5000	161.5	Top left	5830	116.4	93.2	12.9	13.8
		Top right	4820	96.6			
		Middle left	4100	82.4			
		Middle right	4440	89.2			
		Bottom left	4050	81.1			

Diet concentration (ppm)	Eugenol concentration in diet (ppm)		Measured Eugenol in diet (ppm)	% nominal	Mean (%)	SD	%RSD
		Bottom right	4670	93.5			
20000	646	Top left	18480	92.4	92.8	2.1	2.2
		Top right	18590	93.0			
		Middle left	17840	89.2			
		Middle right	18510	92.6			
		Bottom left	18970	94.9			
		Bottom right	18920	94.6			

The diet was homogenous.

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and eugenol is quantified by GC-FID. The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission. The results obtained in this study confirmed that the method is linear, accurate and precise and suitable for the quantification of eugenol in avian diet. The Limit of Quantification was 96.9 µg eugenol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated as part of study number 648-101C (Martin, Nixon, 2007) at concentration levels relevant to the test's results. The method is acceptable for the quantification of eugenol in avian diet.

Water (Ecotoxicology)

KCA 4.1.2/18 (A 2.1.1/09 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/18 (A 2.1.1/09 of this dRR)
Report author	XXXXX
Report year	2008a
Report title	Acute Toxicity of EUGENOL to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test
Report No	37984230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C1 OECD 203 EPA OPPTS 850.1075
Deviations from current test	No information on the daughter ions monitored is presented in the

guideline	report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Eugenol technical grade
CAS number:	97-53-0
Source and lot/batch no.:	Eden Research plc, lot n°95217
Substance content:	98.8% w/w (equivalent to 988 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	in the refrigerator (<5°C), under nitrogen in dark

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer GmbH, lot n°30422
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in the refrigerator (4°C), in dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	not stated
Retention time:	Eugenol: Approx. 17.8 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 1000 mg of test item into 10.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to

ensure that the same volumes of solvent (0.1 ml/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations. The test media were prepared just before introduction of the test organism at the start of the test and just before the test medium renewal (after 48 hours).

Calibration standards: The reference item was used to prepare a stock solution. 26.2 mg of reference item were dissolved in 25 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile/test medium (containing 100 mg DMF/L) (50/50, v/v) to obtain standard solutions in the range from 0.3 to 7 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with acetonitrile to obtain solutions of 100 mg/L of test item. These solutions were diluted with test water (containing 100 mg DMF/L) to obtain fortified samples at a level of 1, 3 and 10 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges between 0.3 – 7 mg/L. Five calibration standards were quantified at low range between 0.30 – 3.0 mg/L and at high range between 1.0 – 7.0 mg/L. The equation to the calibration lines were respectively $206695 x^2 + 243250 x + 8674$ and $74621 x^2 + 705071 x - 314492$, the correlation coefficient R was at least 0.9994 (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.075 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 3 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 1.0, 3.0 and 10.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
1.0	92.0*	32.4	4
3.0	103.3	5.9	4
10.0	96.8	10	4
Overall	100*	5.8*	8*

* The precision test failed 1.0 mg/L (RSD > 20%). Therefore, the results at 1.0 mg/L is not included in overall accuracy/repeatability results.

The data indicates that the accuracy and precision of the method is acceptable at a level of 3.0 mg/L and above. in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set 3.0 mg/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding for the 96 hours test period). After 48 hours of exposure the mean measured test item concentrations were 92% (88 - 111%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 96% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in fish test medium has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 3.0 mg/L for eugenol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in fish test medium.

KCA 4.1.2/19 (A 2.1.1/10 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/19 (A 2.1.1/10 of this dRR)
Report author	XXXXX
Report year	2008b

Report title	Acute Toxicity of EUGENOL to Zebra Fish (<i>Danio rerio</i>) in a 96-hour Semi-static Test
Report No	37983230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Eugenol technical grade
CAS number:	97-53-0
Source and lot/batch no.:	Eden Research plc, lot n°95217
Substance content:	98.8% w/w (equivalent to 988 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	in the refrigerator (<5°C), under nitrogen in dark

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer GmbH, lot n°30422
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in the refrigerator (+4°C), in dark

Analysis parameters

Method type

GC-MS

Instrument:

Agilent 6890 Series Gas Chromatograph with mass spectrometry detector

Analytical column:

HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness

Oven:

60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)

Injection volume

1 µL

Injection mode

Splitless mode

Carrier gas:

Helium

Flow rate:

not stated

Retention time:

Eugenol: Approx. 17.8 min

Monitored ions/transitions

Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 180 g/L was prepared by dissolving 1800 mg of test item into 10.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 ml/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test organism at the start of the test and just before the test medium renewal (after 48 hours).

Calibration standards: The reference item was used to prepare a stock solution. 26.2 mg of reference item were dissolved in 25 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile/test medium (containing 100 mg DMF/L) (50/50, v/v) to obtain standard solutions in the range from 0.5 to 10mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with acetonitrile to obtain solutions of 100 mg/L of test item. These solutions were diluted with test water (containing 100 mg DMF/L) to obtain fortified samples at a level of 1, 3 and 20 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$
Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.5 – 10 mg/L. Six calibration standards were quantified. The equation to the calibration line was $41723 x^2 + 244692 x - 70216$ and the correlation coefficient $R = 0.9988$ (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences >

30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.02 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 3 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 1.0, 3.0 and 20.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
1.0	126.2*	20.0*	6
3.0	93.2	13.3	6
20.0	101.5	6.8	6
Overall	97.3*	10.8*	12*

* The accuracy test failed at 1.0 mg/L (mean recovery >110%). Therefore, the results at 1.0 mg/L are not included in overall accuracy/repeatability results.

The data indicates that the accuracy and precision of the method is acceptable at a level of 3.0 mg/L and above. in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set 3.0 mg/L.

Stability:

Samples were analysed directly after sampling. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding for the 96 hours test period). After 48 hours of exposure the mean measured test item concentrations were 109% (86 - 127%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 109% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in fish test medium has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 3.0 mg/L for eugenol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in fish test medium.

KCA 4.1.2/20 (A 2.1.1/11 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in daphnia test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/20 (A 2.1.1/11 of this dRR)
Report author	Pavié B., Wydra V.
Report year	2008
Report title	Acute Toxicity of EUGENOL to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	37982220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202 EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.3.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Eugenol technical grade
CAS number:	97-53-0
Source and lot/batch no.:	Eden Research plc, lot n°95217
Substance content:	98.8% w/w (equivalent to 988 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	in the refrigerator (<5°C), under nitrogen in dark

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer GmbH, lot n°30422
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in the refrigerator (2 – 10°C), in dark

Analysis parameters

Method type

GC-MS

Instrument:

Agilent 6890 Series Gas Chromatograph with mass spectrometry detector

Analytical column:

HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness

Oven:

60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)

Injection volume

1 µL

Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	not stated
Retention time:	Eugenol: Approx. 17.8 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A stock solution was prepared by dissolving the test item into dimethylformamide (DMF) in order to have a 50.0 mg/L solution. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 ml/L) were added at each test concentration. A control was tested in parallel (culture medium without addition of the test item) and a solvent control (100 mg DMF/L of culture medium).

The test media were prepared just before introduction of the test organism at the start of the test.

Calibration standards: The reference item was used to prepare a stock solution. 25 mg of reference item were dissolved in 25 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile/test medium (containing 100 mg DMF/L) (50/50, v/v) to obtain standard solutions in the range from 0.1 to 3 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with acetonitrile to obtain solutions of 100 mg/L of test item. These solutions were diluted with test water (containing 100 mg DMF/L) to obtain fortified samples at a level of 0.3, 1 and 3 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$
Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges 0.5 – 10 mg/L. Five calibration standards were quantified at low range between 0.10 – 3.0 mg/L. The equation to the calibration line was $189063 x^2 + 299929 x + 4263$ and the correlation coefficient $R = 0.9999$ (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.007 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 1 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 0.3, 1.0 and 5.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
0.3	72.3	30.5	4
1.0	91.3	12.4	4
5.0	108.8	7.1	4
Overall	100*	12.9*	8*

* The precision test failed at 0.3 mg/L (RSD > 20%). Therefore, the results at 0.3 mg/L is not included in overall accuracy/repeatability results.

The data indicates that the accuracy and precision of the method is acceptable at a level of 1.0 mg/L and above. in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set 1.0 mg/L.

Stability:

Samples were analysed directly after sampling. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after the end of 48 hours test period).

After 48 hours of exposure the mean measured test item concentrations were 104% (98 - 111%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 103% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in daphnia test medium has been acceptably validated for linearity, specificity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 1.0 mg/L for eugenol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in daphnia test medium.

KCA 4.1.2/21 (A 2.1.1/12 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of eugenol in daphnia test medium was not previously evaluated at EU level.</p> <p>The GC-MS method for the analysis of eugenol in aqueous test medium has been validated according to the guideline SANCO/3029/99 rev.4.</p> <p>The limit of quantification (LOQ) was determined to be 3.5 µg/L. The limit of detection (LOD) was determined to be 1.05 µg/L.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with ≤ 20% RSD).</p> <p>The method is acceptable for the quantification of eugenol in daphnia test medium.</p>
-------------------	--

Data point:	CA 4.1.2/21 (A 2.1.1/12 of this dRR)
Report author	Egeler P.
Report year	2021
Report title	Eugenol: A Study on the Chronic Toxicity to Daphnia magna [Analytical phase by Schrag K., 2021, Phase ID: 20E13108-01-RADW]
Report No	20GC3DB
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Eugenol is extracted with toluene and final determination was performed by GC-MS, monitoring three ions of m/z >100.

Materials and methods

Analytical grade eugenol was used as test item

Test material

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	ECT, batch number 40002011619
Substance content:	99.74% w/w (equivalent to 997.4 g/kg)
Expiry date of lot/batch:	July 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC with MS 5973 detector in EI mode
Analytical column:	Agilent VF-WAXms, 30 m x 0.32 mm i.d., 0.25 µm film thickness
Oven:	100°C initial temperature, hold for 3 minutes, ramp at 40°C/min to 240°C and hold for 5 minutes
Transfer line temperature:	240°C
Injection volume	2 µL
Injector inlet temperature:	240°C splitless
Carrier gas:	Helium
Flow rate:	1.2 mL/min

Acquisition type: SIM
MS Quad temperature: 150°C
MS Source temperature: 230°C
Retention time: Eugenol: approx.6.9 min
Monitored ions: Eugenol: 164 m/z (for quantification), 149 and 131 m/z (for confirmation)

Sample preparation

The test solution specimens were taken out of the freezer storage ($\leq -18^{\circ}\text{C}$). Before defrosting, formic acid (1% based on the sample volume; e.g. 0.2 mL to a water sample of 20 mL) and toluene (5:1 (v/v) based on the sample volume; e.g. 4 mL to a water sample of 20 mL) were pipetted into the sample vessels. The samples were then defrosted and homogenised for at least 2 min on a Vortex mixer. After phase separation an aliquot of the upper organic phase was transferred into GC vials and used directly for analysis by GC-MS.

Stock solutions and calibration standards

Eugenol Standard Solution:

A stock solution containing 1000 mg/L (S1000) of Eugenol was prepared by pipetting 18.7 μL of the reference item (density: 1.07 g/mL at 25°C) into a 20 mL volumetric flask and adjusting the volume to 20 mL with toluene. From this stock solution standard solutions of 100 mg/L (S100), 10 mg/L (S10) and 1 mg/L (S1) were prepared in toluene.

Fortification Solution:

Fortification solutions were prepared in toluene using microliter syringes and volumetric flasks.

Table 4.1.2/19-1: Preparation of fortification solutions Eugenol

Fortification solution	Solution used for fortification	Volume in mL used	Final dilution volume in mL	Concentration obtained in mg/L
S100	S1000 (stock solution)	1	10	100
S10	S100	1	10	10
S1	S10	1	10	1

Calibration Standard Solutions

Chromatographic external standard solutions were prepared by diluting the standard solutions of the reference item with toluene as described in the following table:

Table 4.1.2/19-2: Preparation of calibration standard solutions

Standard solution	Standard solution used for preparation	Toluene volume added [μL]	Concentration obtained [$\mu\text{g/L}$]
Std 1000 $\mu\text{g/L}$	S1	-	1000
Std 750 $\mu\text{g/L}$	750 μL S1	250	750
Std 500 $\mu\text{g/L}$	500 μL S1	500	500
Std 250 $\mu\text{g/L}$	250 μL S1	750	250
Std 100 $\mu\text{g/L}$	100 μL Std 500 $\mu\text{g/L}$	400	100
Std 50 $\mu\text{g/L}$	100 μL Std 500 $\mu\text{g/L}$	900	50
Std 10 $\mu\text{g/L}$	100 μL Std 100 $\mu\text{g/L}$	900	10
Std 5 $\mu\text{g/L}$	100 μL Std 50 $\mu\text{g/L}$	900	5

Accuracy (recovery) samples

Control samples of aqueous test medium were fortified with fortification solutions as follows:

Table 4.1.2/19-3: Fortification of aqueous test medium with Eugenol

Matrix	Fortification level [$\mu\text{g/L}$]	Control volume sample [mL]	Fortification solution	Volume added [μL]	Formic Acid added [μL]	Toluene added [mL]
Aqueous test medium	3.50	20	S1	70	200	4
	150	20	S100	30	200	4

Calculations

External standard solutions comparable to the concentration expected in specimens were injected before and after a maximum of 4 samples in the analytical sequence. The concentrations were directly calculated from the peak areas of the samples, using the mean peak area of the two bracketing standards as a one-point-calibration.

The concentration of Eugenol in the aqueous test samples in µg/L was calculated as follows:

$$C = \frac{c * V_{Ex}}{V_W}$$

C Concentration of Eugenol in aqueous test specimens in µg/L

c Concentration of Eugenol in final sample extracts, corrected with interspersed standards in µg/L

V_{Ex} Volume of extraction solvent (toluene, 4 mL)

V_W Volume of water sample (20 mL)

Recoveries were calculated by the following equation:

$$Rec = \frac{R_{found}}{R_{fortified}} \cdot 100 \%$$

Rec Recovery in %

R_{found} Analyte determined in µg/L

R_{fortified} Fortification level in µg/L

Confirmation of substance identification

Confirmation method for the eugenol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 164, 149, 131) over the concentration range 5 µg/L to 1000 µg/L, corresponding to sample concentrations of 1.0 to 200 µg/L; eight standards were quantified:

164 m/z	149 m/z	131 m/z
Y = 157.2854 x + 507.4351 R ² = 0.99690 r = 0.99845	Y = 47.4834 x + 109.6673 R ² = 0.99516 r = 0.99758	Y = 41.2751 x + 301.8119 R ² = 0.99793 r = 0.99897

The linearity of the method is considered validated for this range of concentration of eugenol.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, sample fortified spiked with eugenol, aqueous test medium control for each ion monitored for eugenol analyte.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of eugenol. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. The method is considered specific.

Copies of relevant chromatograms are provided for standards of eugenol, sample fortified spiked with eugenol (at LOQ level and nominal content 125 µg/L), aqueous test medium control for each ion monitored.

LOD, LOQ:

The limit of quantification (LOQ) is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ, as confirmed by recovery and precision results, was 3.5 µg/L of eugenol in aqueous test medium samples.

The limit of detection (LOD) was defined as 30% of the limit of quantification as required by guideline SANCO/3029/99 for residues in control samples (1.05 µg/L of eugenol in aqueous test medium samples).

Accuracy, Repeatability (precision):

The accuracy of the method was determined by comparing analysed and nominal concentrations of the target analyte used in the recovery experiments. The precision of the method was calculated as the

relative standard deviation (RSD) of the recovery data at each fortification level.
The following recoveries were obtained with GC-MS for the three ions monitored:

Table 4.1.2/19-4: Recovery results (accuracy and precision)

	Eugenol m/z 164			Eugenol m/z 137			Eugenol m/z 149		
Spike (µg/L)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
3.5	104	0.9	5	100	4.9	5	82	1.3	5
150.0	107	1.7	5	106	2.0	5	107	1.2	5

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

All samples were analysed within 24 h after extraction, therefore the stability of Eugenol in the final extracts was not assessed.

Conclusions

Eugenol is extracted with toluene and final determination was performed by GC-MS, monitoring three ions of m/z >100.

This analytical method for the determination of eugenol content in daphnia test medium has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 3.5 µg/L for eugenol in aqueous test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in daphnia test medium was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4 the method was successfully validated at concentration levels relevant to the test's results.
The method is acceptable for the quantification of eugenol in daphnia test medium.

KCA 4.1.2/22 (A 2.1.1/13 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in algae test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/22 (A 2.1.1/13 of this dRR)
Report author	Meister Werner A., Wydra V.
Report year	2008
Report title	Toxicity of EUGENOL to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test
Report No	37981210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item

supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4 however not enough replicates was tested at each fortification level.

Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.4/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Materials and methods

Test material

Test Item	
Name:	Eugenol technical grade
CAS number:	97-53-0
Source and lot/batch no.:	Eden Research plc, lot n°95217
Substance content:	98.8% w/w (equivalent to 988 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	in the refrigerator (<5°C), under nitrogen in dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	not stated
Retention time:	Eugenol: Approx. 17.8 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 500 g/L was prepared by dissolving 1000 mg of test item into 2.0 mL dimethylformamide (DMF), then this stock solution was diluted with culture medium to a second stock solution of 100 mg of test item/L by dissolving 0.080 mL of the first stock solution in 400 mL of culture medium. Adequate amounts of DMF were added to the culture medium to achieve the same concentrations of solvent (100 mg/L) in each test solution. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to algae. Adequate volumes of the second stock solution were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test organism at the start of the test.

Calibration standards: The test item was used to prepare a stock solution. 50.1mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with test medium (containing 100 mg DMF/L) to obtain standard solutions in the range from 3 to 40 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with culture medium (containing 100 mg DMF/L) to obtain fortified samples at a level of 4, 8 and 60 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges between 3 – 30 mg/L. Five calibration standards were quantified at low range between 3 – 20 mg/L and at high range between 5 – 30 mg/L. The equation to the calibration lines were respectively $4617 x^2 + 498612 x - 764254$ and $3248 x^2 + 543526 x - 1067869$, the correlation coefficient R was at least 0.9991 (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, control solvent samples, control water samples, and water samples spiked with eugenol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.075 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 4 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 4, 8 and 60 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
4*	111.5	1.9	2
8	82.5	4.3	2
60*	114.0	3.7	2

Overall	103	15.5	6
---------	-----	------	---

* Fortified samples.at 4 mg/L and 60 mg/L were slightly below and above the calibration range, however the values were considered reasonable.

Not enough replicates was tested at each fortification level, however the mean recovery result obtained on all the fortification level are well within the range (70 -110%) and the RSD obtained is below the limit value of 20%. The LOQ is therefore set 4.0 mg/L.

Stability:

Samples were analysed directly after sampling; Stability was not formally investigated and, analysis of 96 hours-old test medium demonstrated that the test item is not stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls (containing algae) were taken at the end of the test (after the 96 hours test period). After 96 hours of exposure the mean measured test item concentrations were 55% (22 - 89%) of the nominal values (average for test concentrations of 16, 32 and 64 mg/L; at lower test concentration the measured values were below the LOQ). Thus, during the test period of 96 hours the algae were exposed to mean measured concentrations of 76% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in alga test medium has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 4.0 mg/L for eugenol in algae test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in algae test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4 the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in algae test medium.

KCP 5.1.2/09 (A 2.1.1/14 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/09 (A 2.1.1/14 of this dRR)
Report author	XXXXX
Report year	2008a
Report title	Acute Toxicity of 3AEY to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test
Report No	34301230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference

	item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Eugenol (reference material)
CAS number:	97-53-0
Source and lot/batch no.:	lot n°30422
Active substance content:	99.0% (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in refrigerator, dark

Analysis parameters

Method type:	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	not stated
Retention time:	Eugenol: Approx. 17.8 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to eugenol, this is equivalent to 0.20, 0.40, 0.81, 1.62 and 3.23 mg/L respectively, based on a eugenol content of 3.23% w/w in the test item.

Stock solutions and calibration standards

Eugenol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only eugenol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 5 mg eugenol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L and stirred. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 15, 20 and 100 mg test item/L. With respect to eugenol, this is equivalent to 0.16, 0.48, 0.65 and 3.23 mg/L respectively, based on a eugenol content of 3.23% w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$ Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$ where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges between 0.1 – 5 mg/L. Five calibration standards were quantified at low range between 0.10 – 1.0 mg/L and at high range between 0.5 – 5.0 mg/L. The equation to the calibration lines were respectively $239000 x^2 + 357883.6477 x - 1481$ and $769 x^2 + 862754 x - 2391402$, the correlation coefficient R was at least 0.9995 (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of eugenol, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.04 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 5 mg test item/L, corresponding to 0.163 mg eugenol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 5.0, 15.0, 20.0 and 100 mg/L, corresponding to 0.16, 0.48, 0.65 and 3.23 mg eugenol/L respectively.

Test item fortification level (mg/L)	Accuracy (%)	%RSD	n
5	106	19.1	4
15	103	5.2	4
20	105	16.6	4
100	103	10.6	6
Overall	104	12	18

The data indicates that the accuracy and precision of the method is acceptable at a 5.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The method is accurate and precise at a level of 5.0 mg test item/L and above. The LOQ is therefore set at 5.0 mg test item/L or 0.163 mg eugenol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding for the 96 hours test period). After 48 hours of exposure the mean measured test item concentrations were 78% (63 - 95%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 91% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol by GC-MS.

This analytical method for the determination of eugenol content in fish test medium has been acceptably validated by definition of specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.163 mg/L for eugenol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in fish test medium.

KCP 5.1.2/10 (A 2.1.1/15 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in daphnia test medium was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/10 (A 2.1.1/15 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008b

Report title	Acute Toxicity of 3AEY to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	34302220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202 EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer GmbH, lot n°30422
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in the refrigerator (2 – 10°C), in dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	not stated

Retention time: Eugenol: Approx. 17.8 min
Monitored ions/transitions: Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to eugenol, this is equivalent to 0.20, 0.40, 0.81, 1.62 and 3.23mg/L respectively, based on a eugenol content of 3.23% w/w in the test item.

Stock solutions and calibration standards

Eugenol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only eugenol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 8 mg eugenol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 20 and 100 mg test item/L. With respect to eugenol, this is equivalent to 0.16, 0.65 and 3.23 mg/L respectively, based on a eugenol content of 3.23% w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$
Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges between 0.1 – 8 mg/L. Five calibration standards were quantified at low range between 0.10 – 1.0 mg/L and at high range between 0.5 – 8.0 mg/L. The equation to the calibration lines were respectively $129825 x^2 + 405328 x - 5369$ and $9192 x^2 + 846834 x - 325884$, the correlation coefficient R was at least 0.9984 (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for eugenol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.01 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 5 mg test item/L, corresponding to 0.163 mg eugenol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 5.0, 20.0 and 100 mg/L, corresponding to 0.16, 0.65 and 3.23 mg eugenol/L respectively.

Test item fortification level (mg/L)	Accuracy (%)	%RSD	n
5	106	10.7	4
20	97	3.4	4
100	112	4.9	4
Overall	105	9	12

The data indicates that the accuracy and precision of the method is acceptable at a 5.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The method is accurate and precise at a level of 5.0 mg test item/L and above. The LOQ is therefore set at 5.0 mg test item/L or 0.161 mg eugenol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after the end of 48 hours test period). After 48 hours of exposure the mean measured test item concentrations were 105% (93 - 118%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 100% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in daphnia test medium has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.163 mg/L for eugenol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.
The method is acceptable for the quantification of eugenol in daphnia test medium.

KCP 5.1.2/11 (A 2.1.1/16 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of eugenol in alga test medium was previously evaluated at EU level.
-------------------	--

Data point:

CP 5.1.2/11 (A 2.1.1/16 of this dRR)

Report author	Grade R., Wydra V.
Report year	2008c
Report title	Toxicity of 3AEY to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test
Report No	34303210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Test item	
Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer GmbH, lot n°30422
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2009
Storage conditions:	in the refrigerator (2 – 10°C), in dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL

Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	Not stated
Retention time:	Eugenol: Approx. 17.8 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 1.0, 3.2, 10.0, 32.0 and 100 mg/L. With respect to eugenol, this is equivalent to 0.032, 0.10, 0.32, 1.03 and 3.23 mg/L respectively, based on a eugenol content of 3.23% w/w in the test item.

Stock solutions and calibration standards

Eugenol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only eugenol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.01 to 5 mg eugenol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 1, 10 and 100 mg test item/L. With respect to eugenol, this is equivalent to 0.032, 0.32, and 3.23 mg/L respectively, based on a eugenol content of 3.23% w/w.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a polynomial regression function of the type $y = cx^2 + bx + a$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

b, c = slope

a = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over two ranges between 0.1 – 5 mg/L. Seven calibration standards were quantified at low range between 0.01 – 0.8 mg/L and at high range between 0.5 – 5.0 mg/L. The equation to the calibration line at low range was $121212 x^2 + 169805 x - 2806$, the correlation coefficient R was at least 0.9957 (y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for eugenol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.11 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 10 mg test item/L, corresponding to 0.33 mg eugenol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 1.0, 10.0 and 100 mg/L, corresponding to 0.032, 0.32 and 3.23 mg eugenol/L respectively.

Test item fortification level (mg/L)	Accuracy (%)	%RSD	n
1	321	60.0	4
10	114	8.7	4
100	106	8.5	4
Overall	110	8.9	8

The data indicates that the accuracy and precision of the method is acceptable at a 10.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The method is accurate and precise at a level of 10.0 mg test item/L and above. The LOQ is therefore set at 10.0 mg test item/L or 0.323 mg eugenol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls (containing algae) were taken at the end of the test (after the 96 hours test period). Under the test conditions eugenol was not stable. All reported results in the report amendment are expressed in terms of the geometric mean concentrations of the test item.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of eugenol content by GC-MS.

This analytical method for the determination of eugenol content in alga test medium has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.323 mg/L for eugenol in alga test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in alga test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in alga test medium.

KCP 5.1.2/12 (A 2.1.1/17 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of eugenol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level.</p> <p>The HPLC-UV/DAD analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW was fully validated, according to SANCO/3029/99 rev.4 guidance document.</p> <p>The LOQ of eugenol was 20.28 mg/kg in sugar feeding solution and 100.6 mg/L in water stock solution.</p>
-------------------	--

	Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD). The study is acceptable.
--	---

Data point:	CP 5.1.2/12 (A 2.1.1/17 of this dRR)
Report author	Aversa S.
Report year	2019
Report title	Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document)
Report No	BT081/19
Document No	Not applicable
Guidelines followed in study	SANCO 3029/99/rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Water stock solution

Sample of aqueous stock solution are diluted in acetonitrile to fit within the calibration range prior to analysis by HPLC-DAD.

50% w/v sugar feeding solution

The 50% w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test item

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L) Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L) Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)
Product density:	1.027 kg/L
Expiry date of lot/batch:	October 2020
Storage conditions:	Ambient, dark.

Analytical standard

Name:	Eugenol
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV3232
Active substance content:	99.0%
Expiry date of lot/batch:	May 2022
Storage conditions:	Ambient, dark.

Origin of samples

The method was used to analyse the water stock solution coming from the ecotoxicological study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated

exposure, Pecorari F., 2019) and the sugar feeding solution coming from the ecotoxicological study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees (*Apis mellifera* L.), 10-day feeding laboratory test, Pecorari F., 2019).

Analysis parameters

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile
Gradient:	Isocratic 60% A/ 40% B
Flow rate:	0.6 mL/min
Retention time:	Eugenol: Approx. 1.4 min
Detector wavelength:	210 nm

Sample preparation

Water stock solution

The water stock solutions originate from study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure, Pecorari F., 2019).

The nominal concentration of the test water stock solutions is 100 g product/L at high level and 6.25 g product/L at low level.

The high-level stock solution is diluted with acetonitrile, first 0.4 mL in 10 mL to produce a 4.0 g product/L solution, then 1 mL in 10 mL to produce a final sample of concentration 0.40 g product/L.

The low-level stock solution is diluted with acetonitrile 0.6 mL in 10 mL to produce a final sample of concentration 0.375 g product/L. Eugenol concentration in the diluted stocks is presented below:

ARAW content in dilution (g/L)	Eugenol content in ARAW	Eugenol content in dilution (mg)	Eugenol extract concentration (mg/L)
0.40	3.29% w/v or 32.04 mg/g (density 1.027)	12.82	12.82
0.375		12.02	12.02

Sugar feeding solution

The test sugar feeding solution originates from study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees (*Apis mellifera* L.), 10-day feeding laboratory test, Pecorari F., 2019).

The nominal concentration of the sugar feeding solution is 50000 mg product/kg (equivalent to 50 mg product/g - high level) and 1300 mg product/kg (equivalent to 1.3 mg product/g - low level).

Samples of test feeding solution (1 g) are mixed with water (2 mL) and acetonitrile (10 mL) and sonicated. Phase separation is obtained by the addition of sodium chloride, shaking and settling. The acetonitrile phase is collected and filtered. At that point, extract concentration is 0.13 mg product/mL low level and 5 mg product/mL high level. The low-level extract is analysed directly, while the high-level extract is further diluted (1 mL in 10 mL) to produce an extract concentration of 0.5 mg product/mL prior to analysis by HPLC-DAD.

High- and low-level extract eugenol concentrations are presented below:

ARAW content in extract (g/L)	Eugenol content in ARAW	Eugenol content in dilution (mg)	Eugenol extract concentration (mg/L)
0.13	3.29% w/v or 32.04 mg/g (density 1.027)	4.165	4.165
0.50		16.02	16.02

Blank feeding solutions are subjected to the same procedure to produce a blank matrix extract used for dilution of standards for matrix effect investigation.

Stock solutions and calibration standards

103.8 mg of eugenol analytical standard were weighted in a 10 mL volumetric flask, dissolved and made up to volume with methanol. This Stock Solution was named SS1Eug and had a eugenol concentration of 10276.20 mg/L.

1 mL of this stock was transferred to a 10 mL volumetric flask and diluted to volume with acetonitrile to produce a 1027.62 mg/L stock (identification: SS2 mix³).

Linearity standards – water stock solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (Eugenol 102.762 mg/L). From this stock, five linearity standards spanning the range 1.5 – 36 mg/L were prepared in acetonitrile.

Linearity standards – sugar feeding solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (Eugenol 102.762 mg/L). From this stock, five linearity standards spanning the range 0.51 - 36 mg/L were prepared in acetonitrile.

Recovery and precision samples

Water stock solution

Recovery and precision samples are prepared at two levels in water: 120 000 mg product /L and 3125 mg product /L. These solutions were diluted in acetonitrile to obtain the following concentrations:

High-level accuracy: 1 mL in 10 mL of acetonitrile followed by 0.25 mL in 10 mL to afford a final concentration of 0.3 g product/L.

Low-level accuracy: 1 mL in 10 mL to afford a final concentration of 0.313 g product/L.

High- and low-level accuracy samples eugenol concentrations are presented below:

ARAW content in extract (g/L)	Eugenol content in ARAW	Eugenol content in dilution (mg)	Eugenol extract concentration (mg/L)
0.3	3.29% w/v or 32.04 mg/g (density 1.027)	9.61	9.61
0.3125		10.01	10.01

Sugar feeding solution

A 6329.19 mg product/L stock solution is prepared in water to use as fortification solution for the sugar feeding solution.

Blank sugar feeding solution is fortified levels using the aforementioned stock solution in water to produce fortified samples at 60000 mg product/kg (equivalent to 60 mg product/g - high level) and 650 mg product/kg (equivalent to 0.65 mg product/g - low level). Fortified sugar feeding solutions are extracted as described above to produce extract of concentrations 0.065 g product/L (low-level) and 6.0 g product/L (high level). The low-level extract is analysed directly while the high-level is further diluted 1 mL in 10 mL with acetonitrile.

High- and low-level accuracy samples eugenol concentrations are presented below:

ARAW content in extract (g/L)	Eugenol content in ARAW	Eugenol content in dilution (mg)	Eugenol extract concentration (mg/L)
0.065	3.29% w/v or 32.04 mg/g (density 1.027)	2.08	2.08
0.60		19.22	19.22

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

³ This SS2 mix stock also contains the other two analytes, thymol and geraniol, which are not considered in this assessment and are treated separately.

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Matrix effects:

Matrix effect were investigated by preparing two calibration standards either in matrix (water or sugar feeding solution extract) or in acetonitrile and comparing response for suppression or enhancement. No significant (> 20%) suppression nor enhancement was observed in any of the matrices and calibration standards were prepared in acetonitrile.

Specificity:

The specificity for the three analytes was established by the comparison of the UV Spectrum of the standard solution and a high level recovery solution. In addition, specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific. Copies of relevant chromatograms are provided for standards of active substances (eugenol, thymol and geraniol), blank test medium samples (water and sugar feeding), and matrix samples spiked with actives substances (eugenol, thymol and geraniol).

	Water stock solution		Sugar feeding solution	
Linearity	1.5 – 36.0 mg/L, n = 5 Y = 15.6273x + 4.5099 R = 0.9997		0.5 – 36.0 mg/L, n = 5 Y = 15.9277x – 1.0758 R = 1.0000	
Accuracy	120000 mg prod./L Eq. to 3868 mg eugenol/L	3125 mg prod./L Eq. to 100.6 mg eugenol/L	60000 mg prod/kg Eq. to 1928 mg eugenol/L	650 mg prod./kg Eq. to 20.28 mg eugenol/kg
	101.95%	97.44%	105.2%	101.4%
%RSD	1.41%	0.66%	1.35%	2.77%
LOQ	3125 mg prod./L Eq. to 100.6 mg eugenol/L		650 mg prod./kg Eq. to 20.28 mg eugenol/kg	
LOD	1.5 mg/L (lowest calibration level)		0.5 mg/kg (lowest calibration level)	

The method is accurate and precise for eugenol at a level of 20.28 mg/kg and above in sugar feeding solution and at 100.6 mg/L in water stock solution.

The data indicates that the accuracy and precision of the method is acceptable for the fortification level in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability was not investigated.

Conclusions

Eugenol is extracted from test medium (sugar feeding solution and water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of eugenol content in aqueous stock solution and sugar feeding solution has been acceptably validated by definition of specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 20.28 mg/kg in sugar feeding solution and 100.6 mg/L in water stock solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in aqueous stock solution and sugar feeding solution.

KCP 5.1.2/14 (A 2.1.1/18 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of eugenol in aqueous stock solutions from bee larvae chronic studies was not previously evaluated at EU level.</p> <p>The method for quantification of eugenol in aqueous stock solution used in the 22-day larval toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19).</p> <p>The LOQ of eugenol was 100.6 mg/L in water stock solution.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/14 (A 2.1.1/18 of this dRR)
Report author	Pecorari F.
Report year	2019b
Report title	Effects of ARAW on honeybees (<i>Apis mellifera</i> L.) 22-day larval toxicity test with repeated exposure
Report No	BT060/19
Document No	Not applicable
Guidelines followed in study	OECD Guidance Document, No. 239 "Honey Bee (<i>Apis mellifera</i> L.) Larval Toxicity test, Repeated Exposure (15-Jul-2016)".
Deviations from current test guideline	None.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous stock solution are diluted in acetonitrile to fit within the calibration range prior to analysis by HPLC-DAD.

Although the study report refers to ARAW (alternative name of 3AEY) which contains eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	<p>Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L)</p> <p>Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L)</p> <p>Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)</p>
Product density:	1.027 kg/L

Expiry date of lot/batch: October 2020
Storage conditions: Ambient, dark.
Analytical standard
Name: Eugenol
Source and lot/batch no.: Sigma Aldrich, lot n°BCBV3232
Active substance content: 99.0%
Expiry date of lot/batch: May 2022
Storage conditions: Ambient, dark
Analysis parameters
Method type: HPLC-DAD
Instrument: Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column: Agilent Poroshell 120 2.7 µm SB-C18 3.0 x 50 mm
Column temperature: 30°C
Injection volume: 2 µL
Eluent A: Water with 0.1% trifluoroacetic acid
Eluant B: Acetonitrile
Gradient: Isocratic 60% A/ 40% B
Flow rate: 0.6 mL/min
Retention time: Eugenol: Approx. 1.4 min
Detector wavelength: 210 nm

Method validation

The method for quantification of eugenol in aqueous stock solution used in the 22-day larval toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Eugenol is extracted from test medium (water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of eugenol content is linear, accurate and precise and suitable for the quantification of eugenol in aqueous stock solution. The Limit of Quantification was 100.6 mg/L in water stock solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in aqueous stock solutions from bee larvae chronic studies was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in aqueous stock solutions.

Sugar feeding solution (Ecotoxicology)

KCP 5.1.2/13 (A 2.1.1/19 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The validation of the method for analysis of eugenol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level.
-------------------	--

	<p>The concentrations of the active substances eugenol, geraniol and thymol in feeding solutions were analyzed.</p> <p>The analysis of samples was performed following the analytical method validated in a dedicated GLP study BT081/19, in compliance with the guideline SANCO/3029/99 rev. 4. The LOQ of eugenol was 20.28 mg/kg in sugar feeding solution.</p> <p>The study is acceptable.</p>
--	--

Data point:	CP 5.1.2/13 (A 2.1.1/19 of this dRR)
Report author	Pecorari F.
Report year	2019a
Report title	Chronic oral effects of ARAW on adult worker honeybees <i>Apis mellifera</i> L., 10-day feeding laboratory test
Report No	BT059/19
Document No	Not applicable
Guidelines followed in study	OECD Guideline for the testing on chemicals 245 “Honey bee (<i>Apis mellifera</i> L.), Chronic Oral Toxicity test (10-day feeding test in the laboratory)”.
Deviations from current test guideline	None.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The 50% w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to ARAW (alternative name of 3AEY) which contains eugenol, geraniol and thymol, only eugenol is considered in this summary.

Materials and methods

Test material

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L) Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L) Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)
Product density:	1.027 kg/L
Expiry date of lot/batch:	October 2020
Storage conditions:	Ambient, dark.

Analytical standard

Name:	Eugenol
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV3232
Active substance content:	99.0%
Expiry date of lot/batch:	May 2022
Storage conditions:	Ambient, dark.

Analysis parameters

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with

	Chemstation and software OpenLab CDS ChemStation
	Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 120 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile
Gradient:	Isocratic 60% A/ 40% B
Flow rate:	0.6 mL/min
Retention time:	Eugenol: Approx. 1.4 min
Detector wavelength:	210 nm

Method validation

The method for quantification of eugenol in aqueous stock solution used in the 10-day chronic adult toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Eugenol is extracted from test medium (sugar feeding solution and water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of eugenol content is linear, accurate and precise and suitable for the quantification of eugenol in sugar feeding solution. The Limit of Quantification was 20.28 mg/kg in sugar feeding solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of eugenol in sugar feeding solutions.

Water, buffer solutions,... (Properties)

KCA 4.1.2/25 (A 2.1.1/20 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of eugenol in aqueous and n-octanol solutions was not previously evaluated at EU level.</p> <p>The n-octanol/water partition coefficient of the test item was determined according to OECD guideline 107 and EC method A.8. The test was performed at 3 different ratios of n-octanol and water buffered at 3 different pH values (pH 4, 7 and 9). The determined value of the log of the partition coefficient was within the acceptable range of ± 0.3 log units.</p> <p>The analytical method was validated following SANCO/3029/99, rev.4 with regard to linearity of detector response, precision, accuracy and non-analyte interference of the analytical system.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD).</p> <p>The limit of quantification (LOQ) for eugenol in aqueous phase was 1.055 mg/L.</p> <p>The limit of quantification (LOQ) for eugenol in n-octanol phase was 6.785 mg/L.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CA 4.1.2/25 (A 2.1.1/20 of this dRR)
Report author	Lingott J.
Report year	2020
Report title	Partition coefficient of Eugenol (Shake-Flask Method)
Report No	S20-06643
Document No	Not applicable
Guidelines followed in study	EEC A8, OECD 107
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The contents of eugenol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

Test material

Analytical grade of eugenol was used as test item.

Test material

Test Item

Name:	Eugenol, technical grade
CAS number:	97-53-0
Source and lot/batch no.:	Dr. Ehrenstorfer, lot G1071096,
Substance content:	99.2% w/w (equivalent to 992 g/kg)
Expiry date of lot/batch:	06.04.2026
Storage conditions:	Refrigerated, dark.

Analysis parameters

Method type	HPLC-UV
Instrument:	Agilent 1260 Infinity
Analytical column:	Cadenza 5CD-C18, 150 x 4.6 mm, 5 µm, Imtakt
Column temperature:	40°C
Detector wavelength	282 nm, bandwidth: 4 nm
Injection volume	10 µL
Eluent A:	Acetonitrile
Eluent B:	Ultra-pure water containing 0.1% phosphoric acid
Flow rate:	1 mL/min
Gradient	

Time [min]	% A	% B
0.0	65	35
5.0	65	35
6.0	90	10
8.0	90	10
9.0	65	35
13.0	65	35

Retention time: Eugenol: Approx. 3.4 min

Sample preparation

pH 4, pH 7 and pH 9 buffers are prepared and saturated in n-octanol prior to the test. Correspondingly, samples of n-octanol are saturated with individual buffer solutions.

Stock solutions of concentration approximately 1000 mg/L are individually prepared in buffer-saturated octanol. These stocks are combined with the corresponding octanol-saturated pH 4, pH 7 or pH 9 buffer and equilibrated as part of the test. The concentration of the test item was determined in both phases. To minimize the risk of including traces of n-octanol into the aqueous phases, the n-octanol phase was removed completely before sampling the aqueous phase.

Prior to analysis, the aqueous phase was diluted by a factor of two with acetonitrile, while the octanol phase was diluted by a factor of 100 with acetonitrile. Diluted samples were quantified by HPLC-UV.

Calibration standards preparation

A stock solution containing 1 mg/mL was prepared in acetonitrile. This stock was sequentially diluted in acetonitrile to produce analytical standards within the range 0.5 – 15 mg/L.

Procedural recovery samples

Procedural recovery samples were prepared either in n-octanol or in a specifically prepared buffer mix.

Aqueous phase, low recovery

0.5 mL of a low spike solution containing 2.110 mg/L eugenol in acetonitrile were transferred into a 1.5 mL vial and 0.5 mL buffer mix were added.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of eugenol in the aqueous phase is 1.055 mg/L.

Aqueous phase, high recovery

0.5 mL of a high spike solution containing 5.275 mg/L eugenol in acetonitrile were transferred into a 1.5 mL vial and 0.5 mL buffer mix were added.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of eugenol in the aqueous phase is 2.638 mg/L.

n-Octanol phase, low recovery

To 0.1 mL of a low spike solution containing 678.5 mg/L eugenol in acetonitrile 0.1 mL n-octanol were added. The samples were filled up to a volume of 1 mL with acetonitrile. In a second dilution step 1 mL of this solution was filled up to a final volume of 20 mL with acetonitrile.

This corresponds to a final dilution factor of 100. The sample was directly used for analysis. Five samples were prepared. The nominal content of eugenol in the n-octanol phase is 6.785 mg/L.

n-Octanol phase, high recovery

0.1 mL of a high spike solution containing 1357 mg/L eugenol in acetonitrile were transferred into a vial and 0.1 mL n-octanol were added. The samples were filled up to a volume of 1 mL with acetonitrile. In a second dilution step 0.1 mL of this solution was filled up to a final volume of 1 mL with acetonitrile.

This corresponds to a final dilution factor of 100. The sample was directly used for analysis. Five samples were prepared. The nominal content of eugenol in the n-octanol phase to 13.57 mg/L.

Calculations

The concentration of analyte in the measured sample was calculated by the following equations:

$$C = \left(\frac{(A - b)}{a} \right) \cdot d \cdot \frac{v_{std}}{v_s}$$

Where

$A = a \cdot C + b$ (calibration equation)

A = response ['Area']

b = Y-axis intercept of the calibration curve ['Area']

a = slope of the calibration curve ['Area'/(mg/L)]

C = concentration of analyte in measuring sample [mg/L]

d = dilution factor

v_{std} = injection volume of standards

v_s = injection volume of sample

The amount of analyte in the measuring sample was calculated by the following equations:

$$M = \frac{C \cdot V}{1000 \text{ mL}}$$

with

M = amount of analyte in measuring sample [mg]
 V = volume of the aqueous or n-octanol phase [mL]

The calibration curve was determined from standards of analyte which were measured parallel to the samples.

Findings

Linearity:

The method was found to be linear over the range 0.5 – 15 mg/L for eugenol at each pH. Six calibration standards were quantified. The equations to the calibration line were respectively:

- at pH 4: $0.976174x - 0.002640$ and the correlation coefficient $R = 1.0000$,
- at pH 7: $0.974305x - 0.002799$ and the correlation coefficient $R = 1.0000$,
- at pH 9: $0.972590x - 0.005206$ and the correlation coefficient $R = 1.0000$,

(y = peak area of eugenol, x = concentration of eugenol (in mg/L)).

The linearity of the method is validated for this range of concentration at different pH.

Specificity:

Blank samples of the buffers and n-octanol were prepared as described above and quantified by HPLC-UV. No signal was observed in any of the blank samples at the retention time of eugenol. No interferences were observed.

Copies of relevant chromatograms are provided for standards of eugenol, blank samples containing buffer mix, blank samples containing n-octanol, aqueous phase of sample with buffer pH 4, 7 and 9, n-octanol phase of sample with buffer pH 4, 7 and 9, and recovery samples for n-octanol phase and for aqueous phase.

LOD, LOQ:

The limit of quantification (LOQ) for eugenol in aqueous phase after dilution was 1.055 mg/L (lowest recovery concentration), corresponding 2.110 mg/L in the initial aqueous phase sample.

The limit of quantification (LOQ) for eugenol in n-octanol phase after dilution was 6.785 mg/L (lowest recovery concentration), corresponding 678.5 mg/L in the initial n-octanol phase sample.

The limit of detection (LOD) eugenol was 0.5 mg/L (nominal concentration of the lowest calibration standard).

Accuracy, Repeatability (precision):

Phase	Recovery level (mg/L)	Recovery (%)	%RSD	Horrat value	n
Aqueous	2.110	101.3	0.3	0.03	5
	5.275	101.7	0.3	0.04	5
Overall		101.5	0.4	-	10
n-Octanol	678.5	98.8	4	0.02	5
	1357	99.6	0.1	0.03	5
Overall		99.2	0.5	-	10

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (80 -120%) and the Horrat values obtained are well below the limit value of 1.

Conclusions

The contents of eugenol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

The method for quantification of eugenol in solutions from water/n-octanol partition coefficient testing was fully validated for specificity, linearity, accuracy and precision in both phase (aqueous and n-octanol). The method is acceptable for the quantification of eugenol in water/n-octanol partition coefficient testing solutions.

Assessment and conclusion by applicant:

The validation of the method for analysis of eugenol in aqueous and n-octanol solutions was performed under GLP according to Guideline SANCO/3030/99 rev.4 and was successfully validated. Validation also complies with SANCO/3030/99 rev.5.

The method is acceptable for the quantification of eugenol in physicochemical testing solutions.

A 2.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

GRAPES

KCP 5.2.1/05 (A 2.1.2.1/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method was not previously evaluated at EU level.</p> <p>Grape bunches samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANTE/2020/12830, rev.1. for grapes matrix in the EAS Study S20-06528.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in grapes is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations, except for geraniol and thymol where the blank value was around 50%.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.1.2/05 (A 2.1.2.1/01 of this dRR)
Report author	Chadwick G
Report year	2021a
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to grapevine, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F, 2021, report n°S20-06337-L1]
Report No	S20-06337
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

- Eugenol**

Principle of the method

Eugenol is quantified after extraction from grape samples, by GC-MS (three ions monitored).

The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins

Agroscience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only eugenol is considered in this summary.

Materials and methods

Analytical standard

Name:	Eugenol
CAS No.:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV3232
Active substance content:	99.0%
Expiry date of lot/batch:	May 2022
Storage conditions:	Ambient, dark.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column:	Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven:	100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature:	280°C
Injection volume:	1 µL
Injector temperature:	250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas:	Helium
Flow rate:	1.1 mL/min
Ionisation mode	Electron Impact Ionisation (EI)
Acquisition type:	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Retention time:	Eugenol: approx. 15.8 min
Monitored ions (m/z)	Eugenol: 164 (30 ms dwell) for quantification, 149 (30 ms dwell) and 133 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step.

For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortexed for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortexed for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortexed for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of eugenol: the eugenol reference item (between 2 – 50 mg) was dissolved in acetonitrile

with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of grape were fortified prior to extraction with the fortification solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control grape samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the eugenol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 164) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of eugenol residues in grape and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $792x + 897$, the correlation coefficient R was at least 0.9948 (y = peak area of eugenol, x = concentration of eugenol (in ng/mL)).

The linearity of the method is validated for this range of concentration of eugenol.

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

Specificity:

Specificity was studied by analysis samples of standards of eugenol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with eugenol, treated grape sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of eugenol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of eugenol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with eugenol, treated grape sample for the quantification ions (m/z 164).

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in grape.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the grapes for the quantification ions (m/z 164).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	85	17	6
0.10	93	8	6
Overall	89	13	12

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

Conclusions

Eugenol is quantified after extraction from grape samples, by GC-MS (three ions monitored).

This analytical method for the determination of eugenol content in grapes samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4

requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol in grapes. The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated. The method is acceptable for the quantification of eugenol in grapes.

• Methyleugenol

Principle of the method

Methyleugenol is quantified after extraction from grape samples, by GC-MS (three ions monitored). The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only methyleugenol is considered in this summary.

Materials and methods

Analytical standard

Name:	Methyleugenol
CAS No.:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, lot n°BCCC0277
Active substance content:	99.0%
Expiry date of lot/batch:	July 2022
Storage conditions:	at 4°C, dark.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column:	Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven:	100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature:	280°C
Injection volume:	1 µL
Injector temperature:	250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas:	Helium
Flow rate:	1.1 mL/min
Ionisation mode	Electron Impact Ionisation (EI)
Acquisition type:	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Retention time:	Methyleugenol: approx. 14.5 min
Monitored ions (m/z)	Methyleugenol: 178 (30 ms dwell) for quantification, 163 (30 ms dwell) and 147 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step. For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by

hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of methyleugenol: the methyleugenol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of grape were fortified prior to extraction with the fortification solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control grape samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the methyleugenol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 178) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of methyleugenol residues in grape and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $1013x - 5$, the correlation coefficient R was 0.9953 (y = peak area of methyleugenol, x = concentration of methyleugenol (in ng/mL)).

The linearity of the method is validated for this range of concentration of methyleugenol.

No data was provided for the two confirmation ions monitored (m/z 163 and 147).

Specificity:

Specificity was studied by analysis samples of standards of methyleugenol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with methyleugenol, treated grape sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of methyleugenol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of methyleugenol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with methyleugenol, treated grape sample for the quantification ions (m/z 178).

No data was provided for the two confirmation ions monitored (m/z 163 and 147).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in grape.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the grapes for the quantification ions (m/z 178).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
-----------------------------	----------	---------	---

0.01 (LOQ)	88	16	6
0.10	94	6	6
Overall	91	12	12

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.
No data was provided for the two confirmation ions monitored (m/z 163 and 147).

Conclusions

Methyleugenol is quantified after extraction from grape samples, by GC-MS (three ions monitored). This analytical method for the determination of methyleugenol content in grapes samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for methyleugenol in grapes.
The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated. The method is acceptable for the quantification of methyleugenol in grapes.

KCP 5.2/01 (A 2.1.2.1/02 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyleugenol in grapes was not previously evaluated at EU level.</p> <p>The analytical method has been fully validated for the determination of eugenol, methyl-eugenol, thymol and geraniol in grapes samples according to the SANTE/2020/12830, rev.1. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification was 0.01 mg/kg for eugenol, methyl-eugenol, thymol and geraniol in grapes.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The studies are acceptable.</p>
-------------------	---

Data point:	CP 5.2/01 (A 2.1.2.1/02 of this dRR)
Report author	Driss F
Report year	2021a
Report title	Validation of Residue Method for the Determination of Eugenol, Geraniol, Thymol and Methyl-Eugenol in Grape
Report No	S20-06528
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830, rev.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method was used in Eurofins studies S20-06337 and S20-06526 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which are included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol and methyleugenol are considered in this summary.

Materials and methods

Analytical grade of eugenol and methyleugenol were used as test item.

Test material

Test Standards

Name: Eugenol analytical grade
CAS number: 97-53-0
Source and lot/batch no.: Sigma Aldrich, batch number BCBV3232
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 May 2022
Storage conditions: Ambient

Name: Methyleugenol analytical grade
CAS number: 93-15-2
Source and lot/batch no.: Sigma Aldrich, batch number BCCC0277
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 July 2022
Storage conditions: +4°C

Analysis parameters

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C
Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 15.8 min for eugenol
Approx. 14.5 for methyleugenol
Ionisation mode Electron Impact Ionisation (EI)

Scan type	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Solvent delay	6 min
Ions monitored	Eugenol: 164 # (100 ms dwell), 149 (100 ms dwell), 133 (100 ms dwell) Methyleugenol: 178 # (100 ms dwell), 163 (100 ms dwell), 147 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

An amount of 10 g ± 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol and methyleugenol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, eugenol and methyleugenol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of grapes which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of eugenol in initial grapes samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of grapes on the GC-MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
A _{Solv-Std}	Mean peak area of solvent standard
A _{Matrix-Std}	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
C _{Solv-Std}	Nominal concentration of standard in ng/mL
C _{Matrix-Std}	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of eugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 7 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali1} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
----------------	--------------------------

R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions:

Eugenol: m/z 164, 149 and 133.

Methyleugenol: m/z 178, 163 and 147.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Eugenol (%)		
		Quantification (m/z 164)	Confirmation (m/z 149)	Confirmation (m/z 133)
Grapes	80	(+) 8.3	(-) 1.6	(+) 18.1

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Methyleugenol (%)		
		Quantification (m/z 178)	Confirmation (m/z 163)	Confirmation (m/z 147)
Grapes	80	(+) 5.6	(+) 8.5	(+) 11.7

Matrix suppression or enhancement was $\leq 20\%$ in grapes for eugenol and methyleugenol thus deemed to be insignificant. However, it was shown to be significant for thymol (see below section for thymol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$ ($n = 8$), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for each ion:

Eugenol	<ul style="list-style-type: none"> m/z 164: $y = 489x + 2419$; $r = 0.9977$, $r^2 = 0.9954$ m/z 149: $y = 174x + 162$; $r = 0.9978$, $r^2 = 0.9956$ m/z 133: $y = 89x + 272$; $r = 0.9980$, $r^2 = 0.9959$
Methyleugenol	<ul style="list-style-type: none"> m/z 178: $y = 597x + 918$; $r = 0.9977$, $r^2 = 0.9954$ m/z 163: $y = 179x + 18$; $r = 0.9979$, $r^2 = 0.9957$ m/z 147: $y = 192x + 942$; $r = 0.9977$, $r^2 = 0.9954$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in grape matrix.

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 164 (Proposed for Quantification)							
Grapes	0.01	106; 116; 105; 101; 112	108	6	5	110	6
	0.1	113; 123; 108; 107; 110	112	6	5		
Fragment <i>m/z</i> 149 (Proposed for Confirmation)*							
Grapes	0.01	112; 116; 102; 98; 115	109	7	5	111	7

	0.1	115; 126; 109; 107; 112	114	7	5		
Fragment <i>m/z</i> 133 (Proposed for Confirmation)							
Grapes	0.01	111; 120; 114; 112; 120	115	4	5	114	5
	0.1	112; 125; 108; 108; 109	112	6	5		

* Observable peak was detected in control sample extract (interference around 60% of LOQ). Recoveries are corrected for the mean peak areas of the control sample extracts.

Methyleugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 178 (Proposed for Quantification)							
Grapes	0.01	105; 114; 103; 100; 108	106	5	5	108	6
	0.1	112; 122; 107; 105; 108	111	6	5		
Fragment <i>m/z</i> 163 (Proposed for Confirmation)							
Grapes	0.01	104; 114; 103; 100; 109	106	5	5	108	6
	0.1	111; 122; 107; 105; 108	111	6	5		
Fragment <i>m/z</i> 147 (Proposed for Confirmation)							
Grapes	0.01	104; 116; 100; 99; 109	106	7	5	108	6
	0.1	111; 121; 106; 105; 107	110	6	5		

No observable peak was detected in any control sample extract. Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of eugenol and methyleugenol, control matrix, and control matrix spiked with eugenol and methyleugenol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of eugenol or methyleugenol.

A blank correction was performed with the mean peak area of the control sample(s) used for fortification for only one fragment ion of eugenol (interference around 60% of LOQ). Recoveries and matrix-matched standards were corrected by background subtraction. In addition, three ions were monitored and quantified. The method is specific.

No interference or contamination peak were detected in control samples above 30% LOQ at the same retention time of methyleugenol. In addition, three ions were monitored and quantified. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, and control matrix fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of eugenol and methyleugenol are provided to justify the choice of ions monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1 °C to 10 °C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Eugenol was found to be stable in final extracts of grape for 7 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days* of storage (1 st to	Fortification level	Recovery (%)	Mean recovery	Rel. Std. Dev.	Difference (in %) of recoveries after
--------	---	---------------------	--------------	---------------	----------------	---------------------------------------

	2 nd Injection)	(mg/kg)		(n = 5) (%)	(n = 5) (%)	storage to recoveries before storage in % ^a
Eugenol - Fragment <i>m/z</i> 164 (Proposed for Quantification)						
Grape	0	0.01	106; 116; 105; 101; 112	108	6	(-) 9
	7		86; 100; 97; 88; 117	98	13	
Methyleugenol - Fragment <i>m/z</i> 178 (Proposed for Quantification)						
Grape	0	0.01	105; 114; 103; 100; 108	106	5	(+) 8
	7		86; 99; 96; 87; 116	97	12	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Eugenol and methyleugenol are extracted from grape matrix using acetonitrile and solid-phase extraction clean-up, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity.

This analytical method for the determination of eugenol and methyleugenol content in grape matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol and methyleugenol in grapes.

The method presented herewith is satisfactory and was applied to quantify eugenol and methyleugenol in grapes in Eurofins study S20-06337 and S20-6526 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol and methyleugenol in grapes was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1 and was successfully validated.

The method is acceptable for the quantification of eugenol and methyleugenol in grapes.

KCP 5.2/02 (A 2.1.2.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS Spain).

Comments of zRMS:	<p>The study “<i>Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions</i>” was not previously evaluated at EU level.</p> <p>Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06528. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte.</p> <p>The study is acceptable for the quantification of eugenol and methyleugenol in grapes.</p>
-------------------	---

Data point:	CP 5.2/02 (A 2.1.2.1/03 of this dRR)
Report author	Driss F.
Report year	2021b
Report title	Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions

Report No	S20-06526
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830, rev.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within study n°S20-06528 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol and methyleugenol are considered in this summary.

Materials and methods

Analytical grade eugenol and methyleugenol were used as test item.

Test material

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV3232
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 May 2022
Storage conditions:	Ambient

Name:	Methyleugenol analytical grade
CAS number:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, batch number BCCC0277
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 July 2022
Storage conditions:	+4°C

Analysis parameters

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow	50 mL/min at 0.05 min
Injection volume	1 µL (depending on sensitivity)
Injector temperature	250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:

Approx. 15.8 min for eugenol

Approx 14.5 min for methyleugenol

Ionisation mode

Electron Impact Ionisation (EI)

Scan type

SIM (Selected Ion Monitoring)

Ion source temperature

230°C

Quadrupole temperature

150°C

Solvent delay

6 min

Ions monitored

Eugenol: 164 (100 ms dwell)

Methyleugenol: 178 (100 ms dwell)

Since the method was already validated for specificity, this study only monitored a single ion.

Sample preparation

An amount of 10 g \pm 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μ L of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.1 mg/kg, eugenol and methyleugenol concentration in the final extract was 1.0 μ g/mL.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 μ g/mL for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of grapes which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 μ g/mL (equivalent to 0.0025 to 0.50 mg/kg of eugenol or methyleugenol in initial grapes samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of eugenol and methyleugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by

single extraction and single injection.

Since the method had already been validated, matrix effects, LOQ was not investigated, and a single ion was monitored per analyte.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries ($R_{ProcRec}$) are calculated as follows:

$R_{Rel} (%) \text{ and } R_{ProcRec} (%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{Corrected}$) is calculated as follows:

$R_{Corrected} (%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0 analysis ($P_{Remaining}$) is calculated as follows:

$R_{Remaining} (%) =$	$\frac{R_{Rel_Mean}}{R_{Day0_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
R_{Day0_Mean}	Unrounded mean R_{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Eugenol	m/z 164: $y = 511x + 2149$; $r = 0.9989$, $r^2 = 0.9979$
Methyleugenol	m/z 178: $y = 615x + 3590$; $r = 0.9989$, $r^2 = 0.9978$

The method is linear over the calibration range.

LOQ, LOD:

The LOQ of the method was verified as part of Study n°S20-06528 (Driss 2021a). the LOQ of the method is 0.01 mg/kg.

The LOD of the method was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0 storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The

following recoveries were obtained:

Matrix	Fortification level (mg/kg)		Recovery (%)					Mean (%)	Rel. Std. Dev. (%)
		0 days	43 days	111 days	154 days	280 days			
		Eugenol							
Grape	0.1	85, 81, 79	91	105	110	99	93	13	
		Methyleugenol							
Grape	0.1	73, 73, 79	90	103	108	103	90	17	

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06528 for the quantification of residues of eugenol and methyleugenol in grape matrix was used to assess the stability of residues of eugenol and methyleugenol in grapes. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify eugenol and methyleugenol in grapes.

Assessment and conclusion by applicant:

The method used to verify the stability of eugenol residue in grapes was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06528 and verified as part of this study. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1.
The method is acceptable for the quantification of eugenol and methyleugenol in grapes.

APPLES

KCP 5.1.2/06 (A 2.1.2.1/04 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyl-eugenol residues in apples was not previously evaluated at EU level.</p> <p>Apple fruit samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANCO/3029/99, rev.4 for apple matrix in the EAS Study S20-06529.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in apple is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/06 (A 2.1.2.1/04 of this dRR)
Report author	Chadwick G.
Report year	2021b
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to apple, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F., 2021, report n° S20-06361-L1]
Report No	S20-06361
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

- **Eugenol**

Principle of the method

Eugenol is quantified after extraction from apple samples, by GC-MS (three ions monitored). The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission. Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol is considered in this summary.

Materials and methods

Analytical standard

Name:	Eugenol
CAS No.:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV3232
Active substance content:	99.0%
Expiry date of lot/batch:	May 2022
Storage conditions:	Ambient, dark.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column:	Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven:	100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature:	280°C
Injection volume:	1 µL
Injector temperature:	250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas:	Helium
Flow rate:	1.1 mL/min
Ionisation mode:	Electron Impact Ionisation (EI)
Acquisition type:	SIM (Selected Ion Monitoring)
Ion source temperature:	230°C
Quadrupole temperature:	150°C
Retention time:	Eugenol: approx. 15.4 min
Monitored ions (m/z):	Eugenol: 164 (30 ms dwell) for quantification, 149 (30 ms dwell) and 133 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step. For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of eugenol: the eugenol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of apple were fortified prior to extraction with the fortification

solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control apple samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{End} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the eugenol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 164) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of eugenol residues in apple and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest

analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $151x + 1885$, the correlation coefficient R was at least 0.9993 (y = peak area of eugenol, x = concentration of eugenol (in ng/mL)).

The linearity of the method is validated for this range of concentration of eugenol.

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

Specificity:

Specificity was studied by analysis samples of standards of eugenol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with eugenol, treated apple sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of eugenol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of eugenol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with eugenol, treated apple sample for the quantification ions (m/z 164).

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in apple.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the apples for the quantification ions (m/z 164).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	88	15	8
0.10	85	13	8
Overall	86	14	16

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 149 and 133).

Conclusions

Eugenol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

This analytical method for the determination of eugenol content in apples samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol in apples.

The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol residues in apples was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated.

The method is acceptable for the quantification of eugenol in apples.

- **Methyleugenol**

Principle of the method

Methyleugenol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only methyleugenol is

considered in this summary.

Materials and methods

Analytical standard

Name: Methyleugenol
CAS No.: 93-15-2
Source and lot/batch no.: Sigma Aldrich, lot n°BCCC0277
Active substance content: 99.0%
Expiry date of lot/batch: July 2022
Storage conditions: at 4°C, dark.

Analysis parameters

Method type: GC-MS
Instrument: Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column: Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven: 100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature: 280°C
Injection volume: 1 µL
Injector temperature: 250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas: Helium
Flow rate: 1.1 mL/min
Ionisation mode: Electron Impact Ionisation (EI)
Acquisition type: SIM (Selected Ion Monitoring)
Ion source temperature: 230°C
Quadrupole temperature: 150°C
Retention time: Methyleugenol: approx. 13.0 min
Monitored ions (m/z): Methyleugenol: 178 (30 ms dwell) for quantification, 163 (30 ms dwell) and 147 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step. For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of methyleugenol: the methyleugenol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard

solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of apple were fortified prior to extraction with the fortification solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control apple samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{end} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)

F	Nominal fortification level (mg/kg)
---	-------------------------------------

Confirmation of substance identification

Confirmation method for the methyleugenol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 178) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of methyleugenol residues in apple and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $176x + 968$, the correlation coefficient R was at least 0.9993 (y = peak area of methyleugenol, x = concentration of methyleugenol (in ng/mL)).

The linearity of the method is validated for this range of concentration of methyleugenol.

No data was provided for the two confirmation ions monitored (m/z 163 and 147).

Specificity:

Specificity was studied by analysis samples of standards of methyleugenol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with methyleugenol, treated apple sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of methyleugenol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of methyleugenol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with methyleugenol, treated apple sample for the quantification ions (m/z 178).

No data was provided for the two confirmation ions monitored (m/z 163 and 147).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in apple.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the apples for the quantification ions (m/z 178).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	87	14	8
0.10	86	12	8
Overall	86	13	16

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 163 and 147).

Conclusions

Methyleugenol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

This analytical method for the determination of methyleugenol content in apples samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for methyleugenol in apples.

The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for methyleugenol residues in apples was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated. The method is acceptable for the quantification of methyleugenol in apples.

KCP 5.2/05 (A 2.1.2.1/05 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyleugenol in apples was not previously evaluated at EU level.</p> <p>The method was found to be valid according to the guidance document SANTE/2020/12830, rev.1 for the determination of eugenol, geraniol, thymol and methyl-eugenol in apples with the tested LOQ of 0.01 mg/kg.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for three (3) selected MS fragment ions for eugenol, geraniol, thymol and methyl-eugenol are within 70 – 110% with relative standard deviations \leq 20% and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.</p> <p>The LOQ was successfully established at 0.01 mg/kg in apples for all three ions.</p> <p>The LOD was set at the level of the lowest acceptable calibration standard which is 0.0025 mg/kg.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.2/05 (A 2.1.2.1/05 of this dRR)
Report author	Driss F
Report year	2021c
Report title	Validation of residue method for the determination of eugenol, geraniol, thymol and methyleugenol in apple
Report No	S20-06529
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method was used in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol and methyleugenol are considered in this summary.

Materials and methods

Analytical grade eugenol and methyleugenol were used as test item.

Test material

Test Standards

Name: Eugenol analytical grade
CAS number: 97-53-0
Source and lot/batch no.: Sigma Aldrich, batch number BCBV3232
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 May 2022
Storage conditions: Ambient

Name: Methyleugenol analytical grade
CAS number: 93-15-2
Source and lot/batch no.: Sigma Aldrich, batch number BCCC0277
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 July 2022
Storage conditions: +4°C

Analysis parameters

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C
Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 15.8 min for eugenol
Approx. 14.5 min for eugenol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Eugenol: 164 # (30 ms dwell), 149 (30 ms dwell), 133 (30 ms dwell)
Methyleugenol: 178 # (30 ms dwell), 163 (30 ms dwell), 147 (30 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

An amount of 10 g ± 0.1 g of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken

vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of eugenol and methyleugenol in initial apples samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of apples on the GC-MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
A _{Solv-Std}	Mean peak area of solvent standard
A _{Matrix-Std}	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
C _{Solv-Std}	Nominal concentration of standard in ng/mL
C _{Matrix-Std}	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of eugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Fifty recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 15 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the

bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

With:

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = $A_A - (\text{mean})$ blank peak area
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions.

Eugenol: m/z 164, 149 and 133.

Methyleugenol: m/z 178, 163 and 147.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Eugenol (%)		
		Quantification (m/z 164)	Confirmation (m/z 149)	Confirmation (m/z 133)
Apple (SEU)	80	(+) 65	(+) 71.1	(+) 31.7
Apple (NEU)	80	(-) 11.3	(-) 6.6	(-) 13.3

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Methyleugenol (%)		
		Quantification (m/z 178)	Confirmation (m/z 163)	Confirmation (m/z 147)
Apple (NEU)	80	(+) 29.1	(+) 25.1	(+) 31.6
Apple (SEU)	80	(-) 5.9	(-) 7.1	(-) 5.5

Matrix suppression or enhancement was $\geq 20\%$ in apples for eugenol and methyleugenol thus deemed to be significant. Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$ ($n = 8$), corresponding to analyte concentrations of 0.0025 mg/kg to 0.4 mg/kg in initial apple samples. Linearity was confirmed for each ion:

Eugenol	<ul style="list-style-type: none"> - m/z 164: $y = 68x + 1224$; $r = 0.9975$, $r^2 = 0.9951$ - m/z 149: $y = 24x + 479$; $r = 0.9976$, $r^2 = 0.9951$ - m/z 133: $y = 14x + 412$; $r = 0.9981$, $r^2 = 0.9961$
Methyleugenol	<ul style="list-style-type: none"> - m/z 178: $y = 85x - 275$; $r = 0.9974$, $r^2 = 0.9948$ - m/z 163: $y = 26x - 58$; $r = 0.9974$, $r^2 = 0.9949$ - m/z 147: $y = 28x - 75$; $r = 0.9974$, $r^2 = 0.9949$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in apples matrix.

50 samples at LOQ and 50 samples at 10xLOQ were tested for apples because some background residues in the controls that might be quite variable were expected.

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 164 (Proposed for Quantification)							
Apple	0.01	93, 92, 104, 111, 109, 91, 86, 119, 135, 122, 117, 111, 124, 111, 115, 101, 87, 113, 118, 110, 114, 116, 139, 94, 105, 97, 109, 85, 97, 81, 87, 91, 93, 72, 74, 87, 71, 76, 75, 79, 71, 82, 78, 92, 91, 76, 73, 83, 72, 77	96	19	50	91	20
	0.1	107, 93, 114, 118, 108, 99, 105, 85, 97, 88, 106, 96, 102, 95, 98, 94, 102, 99, 91, 88, 91, 103, 107, 108, 82, 76, 83, 73, 71, 73, 82, 75, 61, 59, 70, 66, 73, 71, 72, 73, 70, 73, 69, 75, 63, 57, 65, 64, 68	85	20	49 [#]		
Fragment <i>m/z</i> 149 (Proposed for Confirmation)							
Apple	0.01	96, 90, 103, 112, 109, 90, 85, 123, 129, 117, 118, 109, 114, 112, 109, 95, 85, 103, 107, 102, 109, 106, 143, 92, 103, 96, 110, 90, 98, 83, 89, 91, 98, 72, 75, 89, 69, 81, 78, 81, 77, 80, 82, 94, 90, 79, 70, 87, 75, 79	95	17	50	91	19
	0.1	108, 93, 116, 120, 108, 101, 106, 86, 99, 89, 108, 98, 103, 96, 98, 94, 103, 100, 92, 88, 91, 104, 109, 108, 65, 67, 74, 66, 68, 81, 67, 74, 75, 75, 77, 71, 58, 67, 68, 73, 86, 88, 76, 56, 81, 79, 71, 67, 78	86	19	49 [#]		
Fragment <i>m/z</i> 133 (Proposed for Confirmation)							
Apple	0.01	99, 99, 90, 104, 110, 89, 88,	104	14	50	98	16

		120, 133, 113, 112, 113, 110, 113, 115, 96, 93, 105, 123, 106, 116, 106, 134, 104, 109, 102, 117, 102, 116, 106, 108, 116, 134, 100, 99, 119, 102, 93, 96, 104, 90, 99, 86, 120, 101, 76, 79, 81, 71, 79					
	0.1	109, 96, 117, 121, 109, 99, 106, 87, 99, 90, 107, 98, 106, 97, 101, 95, 104, 102, 93, 90, 93, 103, 109, 109, 69, 73, 90, 76, 80, 94, 78, 93, 93, 92, 95, 87, 66, 77, 78, 83, 98, 90, 78, 59, 85, 80, 75, 70, 82	92	15	49 #		

Dixon test was used to exclude one value out of the range (around 600%)

Methyleugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 178 (Proposed for Quantification)							
Apple	0.01	91, 88, 99, 108, 105, 90, 86, 116, 135, 116, 115, 105, 117, 104, 102, 93, 79, 103, 109, 99, 103, 107, 127, 81, 98, 94, 105, 86, 95, 83, 91, 91, 94, 75, 77, 87, 72, 77, 78, 82, 76, 83, 77, 94, 92, 77, 75, 83, 72, 76	93	16	50	90	18
	0.1	109, 95, 116, 120, 111, 101, 107, 85, 99, 89, 108, 97, 104, 97, 100, 95, 104, 100, 92, 90, 91, 105, 109, 110, 66, 67, 74, 66, 68, 81, 66, 73, 73, 75, 76, 72, 59, 69, 69, 73, 87, 89, 76, 56, 80, 80, 71, 67, 78	87	19	49 #		
Fragment <i>m/z</i> 163 (Proposed for Confirmation)							
Apple	0.01	91, 89, 98, 108, 104, 89, 87, 115, 136, 114, 114, 105, 120, 105, 104, 94, 79, 104, 108, 101, 104, 108, 129, 84, 97, 95, 106, 86, 95, 82, 90, 90, 95, 76, 78, 87, 72, 77, 77, 82, 76, 82, 77, 94, 92, 76, 74, 83, 70, 76	94	16	50	90	18
	0.1	109, 94, 115, 119, 110, 101, 106, 85, 98, 89, 108, 97, 104, 96, 101, 95, 104, 101, 92, 88, 93, 107, 111, 111, 67, 68, 74, 66, 67, 81, 66, 73, 74, 75, 77, 72, 59, 69, 69, 73, 88, 89, 76, 56, 81, 80, 72, 68, 78	87	19	49#		
Fragment <i>m/z</i> 147 (Proposed for Confirmation)							
Apple	0.01	86, 88, 98, 104, 107, 85, 82, 114, 133, 114, 112, 99, 115, 102, 104, 91, 76, 106, 104, 99, 103, 105, 133, 85, 97, 96, 108, 86, 96, 84, 91, 91, 94, 73, 77, 89, 71, 78, 80, 76, 84, 78, 94, 94, 77, 76, 83, 72, 74	93	16	50	90	18
	0.1	112, 96, 117, 121, 113, 103, 110, 86, 102, 91, 110, 99, 105, 98, 101, 96, 106, 101, 96, 90, 92, 108, 112, 112, 67, 68, 75, 67, 67, 82, 66, 74, 74, 75, 76, 73, 59, 70, 69, 73, 87, 89, 76, 56, 80, 80, 72, 67, 79	88	20	49#		

Dixon test was used to exclude one value out of the range (around 600%)

No observable peak was detected in any control sample extract. Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of eugenol and methyleugenol, control matrix, and control matrix spiked with eugenol and methyleugenol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of eugenol and methyleugenol.

A blank correction was not performed. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, and control matrix fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of eugenol and methyleugenol are provided to justify the choice of ions monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1 °C to 10 °C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Eugenol and methyleugenol were found to be stable in final extracts of apple for 15 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 25) (%)	Rel. Std. Dev. (n = 25) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Eugenol - Fragment m/z 164 (Proposed for Quantification)						
Apple	0	0.01	97, 109, 85, 97, 81, 87, 91, 93, 72, 74, 87, 71, 76, 75, 79, 71, 82, 78, 92, 91, 76, 73, 83, 72, 77	83	12	(-) 16
	15		82, 75, 82, 72, 70, 73, 82, 75, 60, 58, 70, 65, 72, 71, 71, 72, 70, 73, 68, 75, 63, 57, 65, 63, 67	70	10	

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 25) (%)	Rel. Std. Dev. (n = 25) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Methyleugenol - Fragment m/z 178 (Proposed for Quantification)						
Apple	0	0.01	94, 105, 86, 95, 83, 91, 91, 94, 75, 77, 87, 72, 77, 78, 82, 76, 83, 77, 94, 92, 77, 75, 83, 72, 76	84	10	(-) 17
	15		80, 72, 83, 72, 73, 73, 80, 74, 64, 57, 70, 66, 73, 70, 72, 72, 69, 72, 69, 74, 61, 57, 63, 63, 65	70	9	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Eugenol and methyleugenol are extracted from apple matrix using acetonitrile and solid-phase extraction clean-up, then quantified by GC-MS using three separate ions for quantification and confirmation of

method specificity.

This analytical method for the determination of eugenol and methyleugenol content in apple matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol and methyleugenol in apples.

The method presented herewith is satisfactory and was applied to quantify eugenol and methyleugenol in apples in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol and methyleugenol in apples was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of eugenol and methyleugenol in apples.

KCP 5.2/06 (A 2.1.2.1/06 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The study “Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions” was not previously evaluated at EU level.</p> <p>Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06529. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte.</p> <p>The recoveries for each of the analytes at each interval were within 70 – 110%, except at 30 days and 287 days, where the values were in the range 70-123%. The overall mean recoveries were in the range 70-110% and the deviations at 30 days and 287 days were considered not to affect the integrity of the results.</p> <p>The overall mean relative standard deviation covering all testing intervals was $\leq 20\%$ for all analytes.</p> <p>With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the storage samples.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.2/06 (A 2.1.2.1/06 of this dRR)
Report author	Driss F.
Report year	2021d
Report title	Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions
Report No	S20-06527
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate,

sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within study n°S20-06529 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol and methyleugenol are considered in this summary.

Materials and methods

Analytical grade eugenol and methyleugenol were used as test item.

Test material

Test Standards

Name: Eugenol analytical grade
CAS number: 97-53-0
Source and lot/batch no.: Sigma Aldrich, batch number BCBV3232
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 May 2022
Storage conditions: Ambient

Name: Methyleugenol analytical grade
CAS number: 93-15-2
Source and lot/batch no.: Sigma Aldrich, batch number BCCC0277
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: 31 July 2022
Storage conditions: +4°C

Analysis parameters

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C
Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 15.8 min for eugenol
Approx 14.5 min for methyleugenol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Eugenol: 164 (100 ms dwell)
Methyleugenol: 178 (100 ms dwell)
Since the method was already validated for specificity, this study only monitored a single ion.

Sample preparation

An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO_4 , 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.1 mg/kg, eugenol and methyleugenol concentration in the final extract was 1.0 $\mu\text{g/mL}$.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 $\mu\text{g/mL}$ for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 $\mu\text{g/mL}$ (equivalent to 0.0025 to 0.50 mg/kg of eugenol or methyleugenol in initial apple samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of eugenol and methyleugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by single extraction and single injection.

Since the method had already been validated, matrix effects was not investigated, and a single ion was monitored per analyte.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries (R_{ProcRec}) are calculated as follows:

$R_{\text{Rel}} (\%) \text{ and } R_{\text{ProcRec}} (\%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{\text{Corrected}}$) is calculated as follows:

$R_{Corrected} (%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0 analysis ($P_{Remaining}$) is calculated as follows:

$R_{Remaining} (%) =$	$\frac{R_{Rel_Mean}}{R_{Day0_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
R_{Day0_Mean}	Unrounded mean R_{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial apple samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Eugenol	m/z 164: $y = 473x - 2171$; $r = 0.9979$, $r^2 = 0.9957$
Methyleugenol	m/z 178: $y = 569x - 1924$; $r = 0.9978$, $r^2 = 0.9957$

The method is linear over the calibration range.

LOQ, LOD:

The LOQ of the method was verified as part of Study n°S20-06529 (Driss 2021c). the LOQ of the method is 0.01 mg/kg.

The LOD of the method was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0 storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The following recoveries were obtained:

Matrix	Fortification level (mg/kg)			Recovery (%)				
		0 days	30 days	112 days	161 days	202 days	285 days	287 days
	Eugenol							
Apple	0.1	79, 91, 83	109	91	96	114*	100	114*
	Methyleugenol							
Apple	0.1	76, 91, 82	116	91	95	-	105	-

* Reanalysis performed for eugenol only to confirm results obtained at 161 days and 285 days

Matrix	Fortification level (mg/kg)	Mean (%)	Rel. Std. Dev. (%)
Eugenol			
Apple	0.1	97	13
Methyleugenol			
Apple	0.1	94	14

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06529 for the quantification of residues of eugenol and methyleugenol in apple matrix was used to assess the stability of residues of eugenol and methyleugenol in apples. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify eugenol and methyleugenol in apples.

Assessment and conclusion by applicant:

The method used to verify the stability of eugenol and methyleugenol residue in apples was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06529 and verified as part of this study. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1.

The method is acceptable for the quantification of eugenol and methyleugenol in apples.

A 2.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

KCP 5.2/08 (A 2.1.2.2/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyleugenol in body tissue (meat and liver) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination eugenol, methyl-eugenol, thymol and geraniol from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4.</p> <p>The LOQ is the lowest validated fortification level for eugenol, methyl-eugenol, thymol and geraniol and was thus successfully established at 0.01 mg/kg in meat and liver for all selected MS fragment ions or mass transitions.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations $\leq 20\%$ and thereby comply with the standard acceptance criteria of the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.2/07 (A 2.1.2.2/01 of this dRR)
Report author	Driss F
Report year	2021f
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body tissue (meat and liver)
Report No	S20-06625
Document No	Not applicable

Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

• Eugenol

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol is considered in this summary.

Materials and methods

Analytical grade eugenol was used as test item.

Test material

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV3232
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 May 2022
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS																
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)																
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)																
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)																
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz																
Purge Flow	50 mL/min at 0.05 min																
Injection volume	1 µL (depending on sensitivity)																
Injector temperature	250°C																
Column oven temperature programme	<table><tr><th>Step</th><th>Rate [°C/min]</th><th>Temperature [°C]</th><th>Hold time [min]</th></tr><tr><td>1</td><td>-</td><td>100</td><td>2.0</td></tr><tr><td>2</td><td>5.0</td><td>160</td><td>0.0</td></tr><tr><td>3</td><td>15</td><td>250</td><td>0.0</td></tr></table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0	2	5.0	160	0.0	3	15	250	0.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]														
1	-	100	2.0														
2	5.0	160	0.0														
3	15	250	0.0														
Retention time:	Approx. 16.5 min for Eugenol																
Ionisation mode	Electron Impact Ionisation (EI)																
Scan type	SIM (Selected Ion Monitoring)																
Ion source temperature	230°C																
Quadrupole temperature	150°C																
Solvent delay	6 min																
Ions monitored	Eugenol: 164 # (100 ms dwell), 149 (100 ms dwell), 133 (100 ms dwell)																

proposed/used for quantification but any of the ions listed can be used for

quantification or qualification

Sample preparation

Meat: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised meat sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.1 $\mu\text{g/mL}$, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 1.0 $\mu\text{g/mL}$.

Liver: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and $\frac{1}{2}$ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 μL of toluene is added; the supernatant was evaporated to 600 μL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 μL .

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.125 $\mu\text{g/mL}$, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 1.25 $\mu\text{g/mL}$.

Stock solutions and calibration standards

Eugenol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 $\mu\text{g/mL}$. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 $\mu\text{g/mL}$ (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL

$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL
-------------------------	---

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of eugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali,1} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)

F	Nominal fortification level (mg/kg)
---	-------------------------------------

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions: m/z 164, 149 and 133.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Eugenol (%)		
		m/z 164	m/z 149	m/z 133
Meat	80	21.8	95.4	19.3
Liver	80	15.1	12.3	(-) 4

Matrix effects were $\geq \pm 20\%$ and deemed to be significant in meat. For liver, it has been shown to be significant for geraniol (See below section for geraniol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$, corresponding to analyte concentrations of 0.025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver. Linearity was confirmed for each ion.

	Meat	Liver
m/z 164 (GC-MS)	$y = 258x + 1910$ $r = 0.9990, r^2 = 0.9980$	$y = 1768x + 16591$ $r = 0.9989, r^2 = 0.9979$
m/z 149 (GC-MS)	$y = 95x + 148$ $r = 0.9989, r^2 = 0.9978$	$y = 610x + 8714$ $r = 0.9988, r^2 = 0.9975$
m/z 133 (GC-MS)	$y = 56x + 465$ $r = 0.9990, r^2 = 0.9980$	$y = 323x + 13384$ $r = 0.9985, r^2 = 0.9970$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg for meat and liver.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0025 mg/kg in meat and 0.0027 mg/kg in liver.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 164							
Meat	0.01	90; 93; 95; 87; 101	93	6	5	90	9
	0.1	93; 84; 70; 88; 94	86	11	5		
Fragment m/z 149							
Meat	0.01	90; 90; 88; 84; 94	89	4	5	86	9
	0.1	89; 82; 66; 86; 93	83	13	5		
Fragment m/z 133							
Meat	0.01	103; 100; 91; 87; 104	97	8	5	92	11
	0.1	93; 86; 71; 89; 97	87	11	5		

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 164							
Liver	0.01	91; 85; 74; 85; 77	82	8	5	90	10
	0.1	99; 92; 100; 96; 99	97	3	5		
Fragment <i>m/z</i> 149							
Liver	0.01	98; 80; 75; 83; 79	83	11	5	91	11

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
	0.1	99; 93; 102; 96; 100	98	4	5		
Fragment <i>m/z</i> 133							
Liver	0.01	102; 113; 83; 97; 81	95	14	5	94	10
	0.1	95; 93; 94; 92; 94	94	1	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of methyleugenol, control matrix, and control matrix spiked with eugenol. Three fragment ions were monitored.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of eugenol. For all fragment ions, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix meat and liver, therefore showing that the method is highly specific. In addition, three ions were monitored and quantified.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of methyleugenol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for eugenol re-analysed extracts were in the range 70-110% and ± 20 % of the original result for liver but not for meat. For liver, as the acceptance criteria were not fulfilled for geraniol (see above and below section for geraniol), final extracts of meat and liver are considered to be unstable for 17 and 8 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Eugenol - Fragment <i>m/z</i> 164						
Meat	0	0.01	90; 93; 95; 87; 101	93	6	(-)27
	17		64; 68; 51; 83; 74	68	17	
Liver	0	0.01	91; 85; 74; 85; 77	82	8	(+)12
	8		94; 93; 80; 100 ; 94	92	8	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Eugenol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity.

This analytical method for the determination of eugenol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify eugenol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of eugenol in meat and liver.

• **Methyleugenol**

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only methyleugenol is considered in this summary.

Materials and methods

Analytical grade methyleugenol was used as test item.

Test material

Test Standards

Name:	Methyleugenol analytical grade
CAS number:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, batch number BCCC0277
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 July 2022
Storage conditions:	+4°C

Analysis parameters

Method type	GC-MS								
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)								
Analytical column:	Rtx-1701 (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)								
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)								
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz								
Purge Flow	50 mL/min at 0.05 min								
Injection volume	1 µL (depending on sensitivity)								
Injector temperature	250°C								
Column oven temperature programme	<table><tr><th>Step</th><th>Rate [°C/min]</th><th>Temperature [°C]</th><th>Hold time [min]</th></tr><tr><td>1</td><td>-</td><td>100</td><td>2.0</td></tr></table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]						
1	-	100	2.0						

Retention time:	Approx. 16.7 min for methyleugenol
Ionisation mode	Electron Impact Ionisation (EI)
Scan type	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Solvent delay	6 min
Ions monitored	Methyleugenol: 178 # (100 ms dwell), 163 (100 ms dwell), 147 (100 ms dwell) # proposed/used for quantification but any of the ions listed can be used for

quantification or qualification

Sample preparation

Meat: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised meat sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. the contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, methyleugenol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, methyleugenol concentration in the final extract was 1.0 µg/mL.

Liver: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 µL of toluene is added; the supernatant was evaporated to 600 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 µL.

For samples fortified at 0.01 mg/kg, methyleugenol concentration in the final extract was 0.125 µg/mL, and for samples fortified at 0.1 mg/kg, methyleugenol concentration in the final extract was 1.25 µg/mL.

Stock solutions and calibration standards

Methyleugenol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/ mL (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of grapes on the GC-MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
$C_{\text{Solv-Std}}$	Nominal concentration of standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of methyleugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 7 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)

	Methyleugenol
--	----------------------

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 178							
Liver	0.01	105; 100; 87 ;97; 93	96	7	5	103	9
	0.1	114; 105; 111; 105; 113	110	4	5		
Fragment <i>m/z</i> 163							
Liver	0.01	102; 99; 83; 95; 80	92	11	5	100	11
	0.1	113; 105; 110; 105; 112	109	3	5		
Fragment <i>m/z</i> 147							
Liver	0.01	103; 103; 98; 95; 98	99	4	5	103	5
	0.1	110; 102; 108; 103; 110	107	4	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of methyleugenol, control matrix, and control matrix spiked with methyleugenol. Three fragment ions were monitored.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of methyleugenol. For all fragment ions, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix meat and liver, therefore showing that the method is highly specific. In addition, three ions were monitored and quantified.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of methyleugenol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for methyleugenol re-analysed extracts were in the range 70-110% and ± 20 % of the original result. But as the acceptance criteria were not fulfilled for eugenol, geraniol and thymol (see above and below sections for geraniol and thymol), final extracts of meat and liver are considered to be unstable for 17 and 8 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Methyleugenol - Fragment m/z 164						
Meat	0	0.01	93; 104; 96; 84; 107	97	9	(+)12
	17		105; 118; 108; 103; 110	109	5	
Liver	0	0.01	105, 100, 87, 97, 93	96	7	(+)14
	8		110, 113, 92, 120, 111	109	10	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Methyleugenol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity.

This analytical method for the determination of methyleugenol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for methyleugenol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify methyleugenol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for methyleugenol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of methyleugenol in meat and liver.

A 2.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

KCP 5.2/09 (A 2.1.2.3/01 of this dRR)

The DT₉₀ of eugenol and methyleugenol in soil is less than three days and therefore this study is no longer required.

Comments of zRMS:	Accepted.
-------------------	-----------

A 2.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

KCP 5.2/09 (A 2.1.2.4/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyl-eugenol in surface water was not previously evaluated at EU level.</p> <p>The methods were successfully validated and is considered suitable for the determination of residues of thymol, methyl-eugenol, geraniol and eugenol in surface water with an LOQ of 0.1 µg/L. Any of the three ions validated for thymol, geraniol and methyl-eugenol are suitable for quantification and/or confirmation. For eugenol, either of the two HPLC columns are suitable for quantification and/or confirmation.</p> <p>The LOQ was 0.1 µg/L.</p> <p>No significant matrix effects were observed therefore calibration solutions for thymol, methyl-eugenol and geraniol were prepared in solvent.</p> <p>All mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for methyl-eugenol, thymol and geraniol are within 70 – 120% with relative standard deviations ≤ 20%.</p> <p>Satisfactory accuracy and precision results were achieved for eugenol on two HPLC columns of differing chemistry (C₁₈ and pentafluorophenyl), monitoring a single transition; mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for eugenol, are within 70 – 120% with RSD ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/09 (A 2.1.2.4/01 of this dRR)
Report author	Chambers J.
Report year	2020a
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Surface Water
Report No	GW/19/001
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

- **Eugenol**

Principle of the method

Eugenol is quantified by direct injection of water samples by LC-MS/MS.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol is considered in this summary.

Materials and methods

Analytical grade of eugenol was used as test item.

Test material

Test Standards

Name: Eugenol analytical grade

CAS number: 97-53-0
Source and lot/batch no.: Sigma Aldrich, batch number BCBV3232
Substance content: 99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch: May 2022
Storage conditions: Ambient

Analysis parameters

Method type: LC-MS/MS – C18 column
Instrument: HPLC and API 6500 MSD
Analytical column: Acquity UPLC HSS T3 (C18), 100 mm x 2.1 mm, 1.8 µm
Column temperature: 55°C
Flow rate: 0.5 mL/min
Gradient:

Time (min)	Ultra pure water (%)	HPLC grade methanol (%)
0	95	5
2.5	5	95
5.5	5	95
6.5	95	5
7.0	95	5
7.5	95	5

Injection volume: 99 µL
Acquisition type: MRM
Source type: Turbo Ion Spray (ESI)
Retention time: Eugenol: approx. 3.0 min
Monitored ions: Eugenol: m/z 162.9 → 148.0

Method type: LC-MS/MS – PFP column
Instrument: HPLC and API 6500 MSD
Analytical column: Phenomenex Kinetex 1.7u PFP 100A, 100 mm x 2.1 mm, 1.7 µm
Column temperature: 55°C
Flow rate: 0.5 mL/min
Gradient:

Time (min)	Ultra pure water (%)	HPLC grade methanol (%)
0	95	5
3.0	5	95
6.0	5	95
6.1	95	5
7.5	95	5

Injection volume: 99 µL
Acquisition type: MRM
Source type: Turbo Ion Spray (ESI)
Retention time: Eugenol: approx. 3.0 min
Monitored ions: Eugenol: m/z 162.9 → 148.0

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.
An aliquot of 1 mL was transferred to an auto-sampler vial for determination by LC-MS/MS.

Stock solutions and calibration standards

Eugenol Standard Solution: 5 mg of eugenol is accurately weighed and dissolved in acetone to produce a 1000 µg/mL stock solution.

This stock solution is successively diluted to prepare 8 calibration standards spanning the concentration range 0.01 to 2.5 ng/mL in ultra-pure water.

No significant matrix effects were observed therefore calibration solutions for eugenol were prepared in solvent.

Accuracy and repeatability

Two fortification solutions, of concentrations 1 µg/mL and 0.1 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in MeOH:H₂O (1:1 v/v). These fortification solutions are used to produce samples fortified at 0.1 µg/L and 1.0 µg/L.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a 1/x weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f}{m \times V_s} \mu\text{g/L}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

V_s = sample volume

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/L}]}{\text{Amount spiked} [\mu\text{g/L}]} \times 100$$

One ion transition was selected for eugenol.

Confirmation of substance identification

Confirmation method for the eugenol identification was not required as the LC-MS/MS method is highly specific (two different chromatographic column and solvent gradient were studied).

Findings

Linearity:

Linearity was confirmed for the ion transition (m/z 162.9 → 148.0) and the two chromatographic methods over the concentration range 0.03 ng/mL to 2.5 ng/mL, corresponding to sample concentrations of 0.03 µg/L to 2.5 µg/L; six to seven standards were quantified:

Method with C18 column	Method with Pentafluorophenyl column
Y = 561000 x - 11300 (n = 7) R ² = 0.9988 r = 0.9994	Y = 157000x + 454 (n = 6) R ² = 0.9984 r = 0.9992

The linearity of the method is validated for this range of concentration of eugenol.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control surface water, and control surface water spiked with eugenol for each chromatographic method used.

No interference or contamination peak were detected in control samples above 30% LOQ at the same retention time of eugenol. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. The method is considered specific.

Copies of relevant chromatograms are provided for standards of eugenol, control surface water, and control surface water spiked with eugenol for each chromatographic method used. Moreover mass spectra of eugenol are provided to justify the choice of ion transition monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.1 µg/L.

The limit of detection (LOD) was 0.03 µg/L (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in surface water and two chromatographic methods were used.

	Method with C18 column			Method with Pentafluorophenyl column		
Spike (µg/L)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.1	95	15.3	5	113	13.0	5
1	91	9.6	5	112	8.1	5
Overall	93	12.3	10	112	10.2	10

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability of the analyte in solvent and matrix was determined. Eugenol extracts were found to be stable for 22 days when stored at 4-6°C.

Conclusions

Eugenol is quantified by direct injection of water samples by LC-MS/MS (two chromatographic methods used).

This analytical method for the determination of eugenol content in surface water has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. The Limit of Quantification was 0.1 µg/L for eugenol in surface water.

The method presented herewith is satisfactory and can be applied to quantify eugenol in surface water.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol in surface water was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of eugenol in surface water.

• **Methyleugenol**

Principle of the method

Methyleugenol is extracted from surface water via steam distillation and quantified by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only methyleugenol is considered in this summary.

Materials and methods

Analytical grade of methyleugenol was used as test item.

Test material

Test Standards

Name:	Methyleugenol analytical grade
CAS number:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV6695
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	July 2020
Storage conditions:	2-8°C

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at

	20°C/min to 250°C
Transfer line temperature:	280°C
Injection volume:	2 µL
Injector inlet temperature:	250°C Splitless
Carrier gas:	Helium
Flow rate:	2.6 mL/min
Acquisition type:	SIM
Retention time:	Methyleugenol: approx. 6.4 min
Monitored ions:	Methyleugenol: 147, 163 and 178 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

500 mL surface water were transferred to a 500 mL round bottom flask. After fortification, anti-bumping granules were added and 10 mL sample transferred to a liquid-liquid extractor followed by 5 mL of hexane/ethyl acetate (1:1 v/v). The flask was attached to the liquid-liquid extractor, placed on a heating mantle, connected to a condenser and switched on. After the sample reached boiling point and when the vapour was observed to be condensing above the liquid-liquid extractor, the procedure ran for 90 minutes. After cooling, the upper organic layer was transferred by pipette to a graduated tube and 1 mL trimethylpentane added.

The remaining contents of the liquid-liquid extractor were transferred to a second graduated tube and partitioned with 2 mL hexane/ethyl acetate (1:1 v/v) and the upper organic layer was combined with the first. The tube was placed in a dri-block and evaporated until < 1 mL remained. The contents were quantitatively transferred to a 1.0 mL volumetric flask and made up to the mark with hexane/ethyl acetate (1:1 v/v). An aliquot was transferred to an auto-sampler vial for determination by GC-MS.

For recovery samples fortified at 0.1 µg/L and 1.0 µg/L, final extract concentrations are 50 ng/mL and 500 ng/mL, respectively.

Stock solutions and calibration standards

Methyleugenol Standard Solution: 5 mg of methyleugenol is accurately weighed and dissolved in acetone to produce a 1000 µg/mL stock solution.

This stock solution is successively diluted to prepare 8 calibration standards spanning the concentration range 10 to 1500 ng/mL in trimethyl pentane.

No significant matrix effects were observed therefore calibration solutions for methyleugenol were prepared in solvent.

Accuracy and repeatability

Two fortification solutions, of concentrations 1 µg/mL and 10 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile. These fortification solutions are used to produce samples fortified at 0.1 µg/L and 1.0 µg/L. Fortified samples are then extracted and processed as described in sample preparation above. Final extract concentrations are 50 ng/mL and 500 ng/mL for 0.1 µg/L and 1.0 µg/L fortifications, respectively.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a $1/x$ weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f}{m \times V_s} \mu\text{g/L}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

V_s = sample volume

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/L}]}{\text{Amount spiked} [\mu\text{g/L}]} \times 100$$

Three ions were selected for methyleugenol.

Confirmation of impurity identification

Confirmation method for the methyleugenol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 147, 163, 178) over the concentration range 10 ng/mL to 1000 ng/mL, corresponding to sample concentrations of 0.02 $\mu\text{g/L}$ to 2.0 $\mu\text{g/L}$; seven standards were quantified:

147 m/z	163 m/z	178 m/z
Y = 31.95441 x + 65.40118 R ² = 0.99986 r = 0.999902	Y = 27.71935 x + 36.00523 R ² = 0.99995 r = 0.999952	Y = 106.46365 x + 7.28262 R ² = 0.99991 r = 0.999934

The linearity of the method is considered validated for this range of concentration of methyleugenol.

Specificity:

Specificity was studied by analysis samples of standards of methyleugenol, control surface water, and control surface water spiked with methyleugenol for each ion monitored for methyleugenol analyte.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of methyleugenol. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. The method is considered specific.

Copies of relevant chromatograms are provided for standards of methyleugenol, control surface water, and control surface water spiked with methyleugenol for each ion monitored. Moreover a mass spectrum of methyleugenol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.1 $\mu\text{g/L}$.

The limit of detection (LOD) was 0.02 $\mu\text{g/L}$ (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in surface water and three ions were monitored.

	Methyleugenol m/z 147			Methyleugenol m/z 163			Methyleugenol m/z 178		
Spike ($\mu\text{g/L}$)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.1	103	7.9	5	94	7.3	5	96	7.2	5
1	89	4.5	9	88	4.9	9	87	5.0	9
Overall	94	9.2	14	90	6.6	14	91	7.4	14

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability of the analyte in solvent and matrix was determined. Methyleugenol was found stable in solvent for 55 days, and methyleugenol extracts were found to be stable for 22 days when stored at 4-6°C.

Conclusions

The methyleugenol content is extracted in surface water via steam distillation and quantified by GC-MS

(three ions monitored).

This analytical method for the determination of methyleugenol content in surface water has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. The Limit of Quantification was 0.1 µg/L for methyleugenol in surface water.

The method presented herewith is satisfactory and can be applied to quantify methyleugenol in surface water.

Assessment and conclusion by applicant:

The validation of the method for analysis for methyleugenol in surface water was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of methyleugenol in surface water.

A 2.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

KCP 5.2/10 (A 2.1.2.5/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyleugenol in air sampling cartridges was not previously evaluated at EU level.</p> <p>The method was successfully validated and is considered suitable for the determination of residues of thymol, eugenol, methyl-eugenol and geraniol in air with an LOQ of 1.2 µg/m³ according to guidance documents SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute¹ for a period of 7 hours.</p> <p>All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/10 (A 2.1.2.5/01 of this dRR)
Report author	Chambers J.
Report year	2020b
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Air
Report No	TS/19/003
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

- **Eugenol**

Principle of the method

Residues of eugenol were extracted from pre-packed XAD-2 cartridges by sonication with ethyl acetate and final determination was performed by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol is considered in this summary.

Materials and methods

Analytical grade eugenol was used as test item

Test material

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV3232
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	May 2022
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	Agilent CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 12.5°C/min to 215°C and hold for 1 minute
Transfer line temperature:	280°C
Injection volume:	3 µL
Injector inlet temperature:	280°C pulsed-splitless
Carrier gas:	Helium
Flow rate:	2.0 mL/min
Acquisition type:	SIM
Retention time:	Eugenol: approx. 9.5 min
Monitored ions:	Eugenol: 103, 137 and 164 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

If the entire contents of the cartridge are required for analysis, transfer the front and rear segments of the cartridge to a screw-top vial including any glass-wool dividers.

If the front and rear segments of the cartridge are to be analysed separately, transfer the front segment of the cartridge to a screw-top vial including any glass-wool dividers before and after the sorbent, and transfer the rear segments of the cartridge to a screw-top vial including any glass-wool dividers after the sorbent, taking care not to pass the rear section through the front section to avoid contamination.

Add ethyl acetate (5 x 1 mL) extraction solvent to the empty cartridge, collecting all washes into the (front segment) vial. Vortex the sample for 1 minute, sonicate the sample for 10 minutes and vortex the sample for 30 seconds. Transfer an aliquot into an auto-sampler vial and quantify by GC-MS.

For recovery samples fortified at 0.5 µg/cartridge and 5.0 µg/cartridge, final extract concentrations are 100 ng/mL and 1000 ng/mL, respectively.

Stock solutions and calibration standards

Eugenol Standard Solution: 5 mg of eugenol is accurately weighed and dissolved in acetonitrile to produce a 1000 µg/mL stock solution. This stock solution is further diluted in ethyl acetate to produce an intermediate stock from which calibration standards in ethyl acetate are produced.

The calibration solutions span the concentration range 20 to 1300 ng/mL in ethyl acetate (corresponding to 0.24 to 15.6 µg/m³).

No significant matrix effects were observed therefore calibration solutions for eugenol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 200 µg/mL and 20 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile.

The target LOQ for each analyte in air was 1.2 µg/m³. The limit of quantification (LOQ) was taken as the lowest fortification level where an acceptable mean recovery is obtained in the range 70 to 110% with an RSD < 20%.

The rate of air flow was 1.0 L/min for a total duration of 7 hours. This resulted in a volume of 420 L or 0.42 m³ over the 420-minute period, equating to a fortification level of 0.504 µg/cartridge, rounded to 0.50 µg/cartridge (0.42 m³ x 1.2 µg/m³).

The two ends of each cartridge were broken off using a tube cutter. The front segment of each cartridge was fortified at 0.5 µg/cartridge (LOQ) by pipetting 25 µl of the 20 µg/mL fortification solution and at 5 µg/cartridge (10 x LOQ) by pipetting 25 µl of the 200 µg/mL fortification solution.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a 1/x weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f \times D}{m \times 1000} \text{ } \mu\text{g/cartridge}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

D = Dilution factor

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/cartridge}]}{\text{Amount spiked} [\mu\text{g/cartridge}]} \times 100$$

Three ions were selected for eugenol.

Confirmation of substance identification

Confirmation method for the eugenol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 103, 137, 164) over the concentration range 20 ng/mL to 1300 ng/mL, corresponding to sample concentrations of 0.24 µg/m³ to 15.6 µg/m³; eight standards were quantified:

137 m/z	103 m/z	164 m/z
Y = 27.39856 x – 60.68973 R ² = 0.99983 r = 0.99991	Y = 41.36315 x - 95.24692 R ² = 0.99977 r = 0.99988	Y = 150.65434 x – 552.09059 R ² = 0.99972 r = 0.99986

The linearity of the method is considered validated for this range of concentration of eugenol.

Specificity:

Specificity was studied by analysis samples of standards of eugenol, control cartridge (front segment), and cartridge (front and back segment) spiked with eugenol for each ion monitored for eugenol analyte.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of eugenol. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. The method is considered specific.

Copies of relevant chromatograms are provided for standards of eugenol, control cartridge (front segment), and cartridge (front and back segment) spiked with eugenol for each ion monitored. Moreover a mass spectrum of eugenol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours.

The limit of detection (LOD) was 0.15 µg/cartridge based on 30% LOQ (lowest calibration level) and equates to 0.36 µg/m³.

Accuracy, Retention capacity, Repeatability (precision):

Accuracy and repeatability (procedural recoveries) were assessed at two levels of concentrations in cartridge and three ions were monitored.

	Eugenol m/z 103			Eugenol m/z 137			Eugenol m/z 164		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n*
0.5	95	-	2	95	-	2	94	-	2
5.0	85	-	2	85	-	2	85	-	2
Overall	90	8.4	4	90	8.5	4	90	7.9	4

Retention capacity was assessed at two levels of concentrations in cartridge and three ions were monitored.

	Eugenol m/z 103			Eugenol m/z 137			Eugenol m/z 164		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.5	89	6.4	6	80	8.7	6	74	7.8	6
5	96	3.2	6	95	3.5	6	95	3.2	6
Overall	92	6.1	12	88	10.7	12	85	14.4	12

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Extraction efficiency:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with eugenol at 5µg/cartridge (10xLOQ) which achieved mean recovery efficiencies from 6 replicate cartridges of 101%, 102% and 101% for eugenol ions m/z 103, 137 and 164, respectively.

Cartridge stability:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with each analyte at 5 µg/cartridge (10xLOQ) and stored in the dark under ambient conditions for 8 days, which achieved mean recovery efficiencies from three replicate cartridges of 100%, 100% and 100% for eugenol ions m/z 103, 137 and 164, respectively.

Stability:

Solvent standards were shown to be stable in solvent solutions stored for 10 and 65 days at nominally 4°C and extract solutions showed no degradation when stored for at least 18 days.

Conclusions

Eugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored).

This analytical method for the determination of eugenol content in air has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. The Limit of Quantification was 1.2 µg/m³ for eugenol content in air.

The method presented herewith is satisfactory and can be applied to quantify eugenol in air via air

sampling cartridges.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol in air sampling cartridges was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of eugenol in air via air sampling cartridges.

- **Methyleugenol:**

Principle of the method

Residues of methyleugenol were extracted from pre-packed XAD-2 cartridges by sonication with ethyl acetate and final determination was performed by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only methyleugenol is considered in this summary.

Materials and methods

Analytical grade methyleugenol was used as test item.

Test material

Test Standards

Name:	Methyleugenol analytical grade
CAS number:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV6695
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	July 2020
Storage conditions:	2-8°C

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	Agilent CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 12.5°C/min to 215°C and hold for 1 minute
Transfer line temperature:	280°C
Injection volume:	3 µL
Injector inlet temperature:	280°C pulsed-splitless
Carrier gas:	Helium
Flow rate:	2.0 mL/min
Acquisition type:	SIM
Retention time:	Methyleugenol: approx.8.0 min
Monitored ions:	Methyleugenol: 105, 163 and 178 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

If the entire contents of the cartridge are required for analysis, transfer the front and rear segments of the cartridge to a screw-top vial including any glass-wool dividers.

If the front and rear segments of the cartridge are to be analysed separately, transfer the front segment of the cartridge to a screw-top vial including any glass-wool dividers before and after the sorbent, and transfer the rear segments of the cartridge to a screw-top vial including any glass-wool dividers after the sorbent, taking care not to pass the rear section through the front section to avoid contamination.

Add ethyl acetate (5 x 1 mL) extraction solvent to the empty cartridge, collecting all washes into the (front segment) vial. Vortex the sample for 1 minute, sonicate the sample for 10 minutes and vortex the sample for 30 seconds. Transfer an aliquot into an auto-sampler vial and quantify by GC-MS. For recovery samples fortified at 0.5 µg/cartridge and 5.0 µg/cartridge, final extract concentrations are 100 ng/mL and 1000 ng/mL, respectively.

Stock solutions and calibration standards

Methyleugenol Standard Solution: 5 mg of methyleugenol is accurately weighed and dissolved in acetonitrile to produce a 1000 µg/mL stock solution. This stock solution is further diluted in ethyl acetate to produce an intermediate stock from which calibration standards in ethyl acetate are produced.

The calibration solutions span the concentration range 20 to 1300 ng/mL in ethyl acetate (corresponding to 0.24 to 15.6 µg/m³).

No significant matrix effects were observed therefore calibration solutions for methyleugenol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 200 µg/mL and 20 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile.

The target LOQ for each analyte in air was 1.2 µg/m³. The limit of quantification (LOQ) was taken as the lowest fortification level where an acceptable mean recovery is obtained in the range 70 to 110% with an RSD < 20%.

The rate of air flow was 1.0 L/min for a total duration of 7 hours. This resulted in a volume of 420 L or 0.42 m³ over the 420-minute period, equating to a fortification level of 0.504 µg/cartridge, rounded to 0.50 µg/cartridge (0.42 m³ x 1.2 µg/m³).

The two ends of each cartridge were broken off using a tube cutter. The front segment of each cartridge was fortified at 0.5 µg/cartridge (LOQ) by pipetting 25 µl of the 20 µg/mL fortification solution and at 5 µg/cartridge (10 x LOQ) by pipetting 25 µl of the 200 µg/mL fortification solution.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a 1/x weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f \times D}{m \times 1000} \text{ } \mu\text{g/cartridge}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

D = Dilution factor

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/cartridge}]}{\text{Amount spiked} [\mu\text{g/cartridge}]} \times 100$$

Confirmation of impurity identification

Confirmation method for the methyleugenol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 105, 163, 178) over the concentration range 20 ng/mL to 1300 ng/mL, corresponding to sample concentrations of 0.24 µg/m³ to 15.6 µg/m³; eight standards were quantified:

105 m/z	163 m/z	178 m/z
Y = 19.32183 x – 22.44889 R ² = 0.99989 r = 0.99994	Y = 50.32346 x - 76.7629 R ² = 0.99995 r = 0.99997	Y = 194.04563 x – 432.53512 R ² = 0.9999 r = 0.99994

The linearity of the method is considered validated for this range of concentration of methyleugenol.

Specificity:

Specificity was studied by analysis samples of standards of methyleugenol, control cartridge (front segment), and cartridge (front and back segment) spiked with methyleugenol for each ion monitored for methyleugenol analyte.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of methyleugenol. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. The method is considered specific.

Copies of relevant chromatograms are provided for standards of methyleugenol, control cartridge (front segment), and cartridge (front and back segment) spiked with methyleugenol for each ion monitored. Moreover a mass spectrum of methyleugenol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours.

The limit of detection (LOD) was 0.15 µg/cartridge based on 30% LOQ (lowest calibration level) and equates to 0.36 µg/m³.

Accuracy, Retention capacity, Repeatability (precision):

Accuracy and repeatability (procedural recoveries) were assessed at two levels of concentrations in cartridge and three ions were monitored.

	Methyleugenol m/z 105			Methyleugenol m/z 163			Methyleugenol m/z 178		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n*
0.5	80	-	2	84	-	2	83	-	2
5.0	85	-	2	85	-	2	85	-	2
Overall	83	3.9	4	84	1.3	4	90	7.9	4

Retention capacity was assessed at two levels of concentrations in cartridge and three ions were monitored.

	Methyleugenol m/z 105			Methyleugenol m/z 163			Methyleugenol m/z 178		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.5	86	8.0	6	84	0.9	6	83	1.0	6
5	82	1.2	6	82	1.1	6	82	1.1	6
Overall	84	6.0	12	83	1.7	12	83	1.3	12

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Extraction efficiency:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with methyleugenol at 5µg/cartridge (10xLOQ) which achieved mean recovery efficiencies from 6 replicate cartridges of 97%, 97% and 97% for methyleugenol ions m/z 105, 163 and 178, respectively.

Cartridge stability:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with each analyte at 5 µg/cartridge (10xLOQ) and stored in the dark under ambient conditions for 8 days, which achieved mean recovery efficiencies from three replicate cartridges of 98%, 97% and 98% for methyleugenol ions m/z 105, 163 and 178, respectively.

Stability:

Solvent standards were shown to be stable in solvent solutions stored for 10 and 65 days at nominally 4°C and extract solutions showed no degradation when stored for at least 18 days.

Conclusions

Methyleugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored).

This analytical method for the determination of methyleugenol content in air has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. The Limit of Quantification was 1.2 µg/m³ for methyleugenol content in air.

The method presented herewith is satisfactory and can be applied to quantify methyleugenol in air via air sampling cartridges.

Assessment and conclusion by applicant:

The validation of the method for analysis for methyleugenol in air sampling cartridges was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of methyleugenol in air via air sampling cartridges.

A 2.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

KCP 5.2/07 (A 2.1.2.6/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for eugenol and methyleugenol in body fluids (plasma and urine) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination of eugenol, geraniol, thymol and methyl-eugenol on plasma and urine from the tested LOQ of 0.01 mg/L up to 0.1 mg/L according to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev.4.</p> <p>The LOQ is 0.01 mg/kg for eugenol, geraniol, thymol and methyl-eugenol for plasma and urine.</p> <p>All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.2/07 (A 2.1.2.6/01 of this dRR)
Report author	Driss F.
Report year	2021e
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body fluid (plasma and urine)
Report No	S20-06626
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17

Deviations from current test guideline None

Previous evaluation No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

• Eugenol

Principle of the method

In brief, samples of body fluids (plasma and urine) were extracted with acetonitrile. A salt mixture containing magnesium sulphate sodium chloride (urine) or magnesium sulphate, sodium chloride and sodium citrate (plasma) was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only eugenol is considered in this summary.

Materials and methods

Analytical grade eugenol was used as test item.

Test material

Test Standards

Name:	Eugenol analytical grade
CAS number:	97-53-0
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV3232
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 May 2022
Storage conditions:	Ambient

Analysis parameters

Method type GC-MS

Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)

Analytical column: Rtx-1701 (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)

And: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek) for confirmatory method of eugenol in plasma

Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)

Injection mode Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz

Purge Flow 50 mL/min at 0.05 min

Injection volume 1 µL (depending on sensitivity)

Injector temperature 250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 16.5 min for Eugenol

Ionisation mode Electron Impact Ionisation (EI)

Scan type SIM (Selected Ion Monitoring)

Ion source temperature 230°C

Quadrupole temperature 150°C

Solvent delay 6 min

Ions monitored Eugenol: 164 # (100 ms dwell), 149 (100 ms dwell), 133 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

Urine: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised urine sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 10 mL acetonitrile was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

The supernatant was transferred into a polypropylene tube and 2 tubes containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL was transferred a centrifuge tube and 100 µL of toluene is added. The supernatant was evaporated down to 300 µL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 800 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 0.75 µg/mL.

Plasma: An amount of $5 \text{ g} \pm 0.1 \text{ g}$ of homogenised plasma sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. The centrifuge tube was capped and vortexed for 5 min. For extraction, exactly 5.0 mL of acetonitrile were added. The centrifuge tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 1 minute. ½ citrate extraction tube containing 4 g of magnesium sulfate, 1 g of sodium chloride was added and the tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 5 minutes. The sample tube was centrifuged for 10 minutes at 4000 rpm at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 3.0 mL is transferred a centrifuge tube and 50 µL of toluene is added; the supernatant was evaporated to 300 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 400 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 0.75 µg/mL.

Stock solutions and calibration standards

Eugenol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of matrix which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0027 mg/kg to 0.532 mg/kg in both matrices).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix plasma and urine on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL

$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL
-------------------------	---

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of eugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali1} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000133 for plasma and urine	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)

F	Nominal fortification level (mg/kg)
---	-------------------------------------

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ions by GC/MS plus one ion transition by LC-MS/MS for plasma and by detection of three ions by GC/MS for urine.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Eugenol (%)		
		m/z 164	m/z 149	m/z 133
Plasma	80	(+) 34.8	(+) 25.5	(+) 8.3
Urine	80	(+) 39	(+) 33.3	(+) 41

For plasma and urine, matrix effects on the detection of Eugenol in extracts were found to be significant ($\geq 20\%$). Therefore matrix-matched standards were used for quantification.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$, corresponding to analyte concentrations of 0.0027 mg/kg to 0.532 mg/kg in plasma and urine. Linearity was confirmed for each ion.

	Plasma	Urine
m/z 164 (GC-MS)	$y = 451x + 4154$ $r = 0.9986, r^2 = 0.9972$	$y = 364x + 4712$ $r = 0.9991, r^2 = 0.9982$
m/z 149 (GC-MS)	$y = 157x + 4316$ $r = 0.9979, r^2 = 0.9957$	$y = 132x + 2377$ $r = 0.9975, r^2 = 0.9951$
m/z 133 (GC-MS)	$y = 0.64x + 6$ $r = 0.9974, r^2 = 0.9949$	$y = 65x + 1711$ $r = 0.9988, r^2 = 0.9975$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg for Plasma and urine.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0027 mg/kg in plasma and urine.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in plasma and urine matrix.

Eugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 164							
Plasma	0.01	74, 94, 104, 70, 100	88	18	5	91	14
	0.1	81, 93, 94, 109, 92	94	11	5		
Urine	0.01	106, 101, 113, 90, 109	104	9	5	98	9
	0.1	98, 88, 87, 100, 90	93	6	5		
Fragment m/z 149							
Plasma	0.01	94, 98, 104, 73, 100	94	13	5	96	11
	0.1	83, 96, 97, 113, 97	97	11	5		
Urine	0.01	105, 96, 102, 94, 106	101	5	5	97	7
	0.1	96, 90, 86, 101, 90	93	6	5		
Fragment m/z 133							
Plasma	0.01	84; 113; 97; 72; 105	94	17	5	98	19
	0.1	83; 90; 130; 96; 113	102	19	5		
Urine	0.01	95, 107, 106, 83, 106	99	11	5	97	9
	0.1	98, 90, 88, 103, 90	94	7	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of eugenol, control matrix, and control matrix spiked with eugenol. Three fragment ions were monitored.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the eugenol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix plasma and urine, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of eugenol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Eugenol was found to be stable in final extracts of plasma and urine for 8 and 10 days, respectively, when stored at typically 1°C to 10°C in the dark. However, the mean recovery value for geraniol (see below section for geraniol) in urine re-analysed extracts were out of the range of $\pm 20\%$ of the original results. Therefore, samples should be analysed as quickly as possible after extraction.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Eugenol - Fragment m/z 149						
Plasma	0	0.01	74, 94, 104, 70, 100	88	18	11
	8		84, 96, 109, 87, 115	98	14	
Urine	0	0.01	124; 107; 103; 114; 130	116	10	4
	10		113; 97; 101; 106; 140	111	15	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Eugenol is extracted from plasma or urine matrix using acetonitrile, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity.

This analytical method for the determination of eugenol content in body fluid matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for eugenol in both plasma and urine.

The method presented herewith is satisfactory and can be applied to quantify eugenol in plasma and urine.

Assessment and conclusion by applicant:

The validation of the method for analysis for eugenol in body fluids (plasma and urine) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of eugenol in plasma and urine.

- **Methyleugenol**

Principle of the method

In brief, samples of body fluids (plasma and urine) were extracted with acetonitrile. A salt mixture containing magnesium sulphate sodium chloride (urine) or magnesium sulphate, sodium chloride and sodium citrate (plasma) was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) prior to quantification.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only methyleugenol is considered in this summary.

Materials and methods

Analytical grade methyleugenol was used as test item.

*Test material***Test Standards**

Name:	Methyleugenol analytical grade
CAS number:	93-15-2
Source and lot/batch no.:	Sigma Aldrich, batch number BCCC0277
Substance content:	99.0% w/w (equivalent to 990 g/kg)
Expiry date of lot/batch:	31 July 2022
Storage conditions:	+4°C

Analysis parameters

Method type	GC-MS																
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)																
Analytical column:	Rtx-1701 (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)																
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)																
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz																
Purge Flow	50 mL/min at 0.05 min																
Injection volume	1 µL (depending on sensitivity)																
Injector temperature	250°C																
Column oven temperature programme	<table><tr><th>Step</th><th>Rate [°C/min]</th><th>Temperature [°C]</th><th>Hold time [min]</th></tr><tr><td>1</td><td>-</td><td>100</td><td>2.0</td></tr><tr><td>2</td><td>5.0</td><td>160</td><td>0.0</td></tr><tr><td>3</td><td>15</td><td>250</td><td>0.0</td></tr></table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0	2	5.0	160	0.0	3	15	250	0.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]														
1	-	100	2.0														
2	5.0	160	0.0														
3	15	250	0.0														
Retention time:	Approx. 16.7 min for Methyleugenol																
Ionisation mode	Electron Impact Ionisation (EI)																
Scan type	SIM (Selected Ion Monitoring)																
Ion source temperature	230°C																
Quadrupole temperature	150°C																
Solvent delay	6 min																
Ions monitored	Methyleugenol: 178# (100 ms dwell), 163 (100 ms dwell), 147 (100 ms dwell)																

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

Urine: An amount of 10 g ± 0.1 g of homogenised urine sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 10 mL acetonitrile was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

The supernatant was transferred into a polypropylene tube and 2 tubes containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL was transferred a centrifuge tube and 100 µL of toluene is added. The supernatant was evaporated down to 300 µL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 800 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, methyleugenol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, methyleugenol concentration in the final extract was 0.75 µg/mL.

Plasma: An amount of 5 g ± 0.1 g of homogenised plasma sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. The centrifuge tube was capped and vortexed for 5 min. For extraction, exactly 5.0 mL of acetonitrile were added. The centrifuge tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 1 minute. ½ citrate extraction tube containing 4 g of magnesium sulfate, 1 g of sodium chloride was added and the tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 5 minutes. The sample tube was centrifuged for 10 minutes at 4000 rpm at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 3.0 mL is transferred a centrifuge tube and 50 µL of toluene is added; the supernatant was evaporated to 300 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 400 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Methyleugenol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of matrix which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0027 mg/kg to 0.532 mg/kg in both matrices).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix plasma and urine on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of methyleugenol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 107 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C _A	Concentration of analyte in final extract (ng/mL)
A _A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000133 for plasma and urine	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions by GC/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Methyleugenol (%)		
		m/z 178	m/z 163	m/z 147
Plasma	80	(+)15.1	(+)0.1	(+)9.2
Urine	80	(+)24.8	(+)20.2	(-) 10.5

Matrix effects on the detection of Methyleugenol in urine extracts were found to be significant ($\geq 20\%$), but not in plasma extracts. However, as matrix effects on the detection of eugenol and geraniol (see above and in below section for geraniol) in plasma extracts were found to be significant ($\geq 20\%$), matrix-matched standards were used for quantification.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$, corresponding to analyte concentrations of 0.0027 mg/kg to 0.532 mg/kg in plasma and urine. Linearity was confirmed for each ion.

	Plasma	Urine
m/z 178 (GC-MS)	$y = 570x + 2162$ $r = 0.9985, r^2 = 0.9970$	$y = 438x + 1712$ $r = 0.9991, r^2 = 0.9982$
m/z 163 (GC-MS)	$y = 169x + 1193$ $r = 0.9986, r^2 = 0.9973$	$y = 131x + 1225$ $r = 0.9989, r^2 = 0.9978$
m/z 147 (GC-MS)	$y = 184x + 3172$ $r = 0.9985, r^2 = 0.9970$	$y = 139x + 567$ $r = 0.9993, r^2 = 0.9987$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg for Plasma and urine.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0027 mg/kg in plasma and urine.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in plasma and urine matrix.

Methyleugenol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 178							
Plasma	0.01	85, 96, 110, 69, 105	93	18	5	94	14
	0.1	84, 92, 111, 92	95	10	5		
Urine	0.01	107, 102, 110, 88, 109	103	9	5	99	9
	0.1	101, 90, 88, 105, 91	95	8	5		
Fragment m/z 163							
Plasma	0.01	87, 83, 99, 72, 110	90	16	5	92	13
	0.1	84, 91, 94, 109, 91	94	10	5		
Urine	0.01	106, 100, 100, 81, 109	99	11	5	96	10
	0.1	97, 89, 86, 100, 88	92	7	5		
Fragment m/z 147							
Plasma	0.01	79, 87, 103, 66, 74	82	17	5	88	15
	0.1	83, 91, 94, 112, 93	95	11	5		
Urine	0.01	109, 97, 87, 82, 90	93	11	5	94	9
	0.1	101, 91, 89, 102, 92	95	6	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of methyleugenol, control matrix, and control matrix spiked with methyleugenol. Three fragment ions were monitored.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of methyleugenol. For all fragment ions or mass transition, the samples showed no

significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix plasma and urine, therefore showing that the method is highly specific. In addition, three ions were monitored and quantified.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of methyleugenol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 107 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Methyleugenol was found to be stable in final extracts of plasma and urine for 8 and 10 days, respectively, when stored at typically 1°C to 10°C in the dark. However, the mean recovery value for geraniol (see below section for geraniol) in urine re-analysed extracts were out of the range of ± 20 % of the original results. Therefore, samples should be analysed as quickly as possible after extraction.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Methyleugenol - Fragment m/z 178						
Plasma	0	0.01	85, 96, 110, 69, 105	93	18	4
	8		84, 95, 113, 82, 109	97	15	
Urine	0	0.01	118, 102, 105, 103, 107	107	6	12
	10		103, 80, 98, 100 [#]	94	14	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection)

[#] Dixon test was performed to exclude one outlier value

Extraction efficiency:

Not required as part of this study.

Conclusions

Methyleugenol is extracted from plasma or urine matrix using acetonitrile, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity.

This analytical method for the determination of methyleugenol content in body fluid matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for methyleugenol in both plasma and urine.

The method presented herewith is satisfactory and can be applied to quantify methyleugenol in plasma and urine.

Assessment and conclusion by applicant:

The validation of the method for analysis for methyleugenol in body fluids (plasma and urine) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of methyleugenol in plasma and urine.

KCP 5.2/08 (A 2.1.2.6/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	See point KCP 5.2/08 (A 2.1.2.2/01)
-------------------	-------------------------------------

Please note that a study on the validation of the analytical method for eugenol and methyleugenol in body tissue (meat and liver) is presented under point KCP 5.2/08 (A 2.1.2.2/01) in this submission.

A 2.1.2.7 Other Studies/ Information

No new or additional studies have been submitted.

A 2.2 Analytical methods for geraniol

A 2.2.1 Methods used for the generation of pre-authorization data (KCP 5.1)

GRAPES (Residues)

KCP 5.1.2/01 (A 2.2.1/01 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in 3AEY has already been evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/01 (A 2.2.1/01 of this dRR)
Report author	Bailey A.
Report year	2007
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern Europe (2006 – 2007)
Report No	AF/10728/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev 4
Deviations from current test guideline	The calibration range does not extend to 30% of the LOQ; Although three ions were monitored, except for specificity, validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol is determined and quantified using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard. The method allows for separate quantification of free and encapsulated geraniol.

Although the study refers to the analysis of eugenol, geraniol and thymol, only geraniol is considered in this assessment.

Test material

Test material

Test item	
Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature
Name:	Geraniol technical
CAS number:	89-83-8
Source and lot/batch no.:	Eden research plc, lot n°96904, purity 98.2%
Expiry date:	April 2008

Storage conditions: In a refrigerated dessicator

Reference material

Name: Geraniol
CAS number: 89-83-8
Source and lot/batch no.: Fluka, lot n°1151550, purity 99.0%
Expiry date: February 2008
Storage conditions: In a refrigerated dessicator

Analysis parameters

Method type: GC-FID
Instrument: GC, 5890 (Hewlett Packard) with flame ionisation detector (FID)
Analytical column: Zebron ZB-5, 30 m x 0.32 mm, 1.0 µm film thickness or equivalent
Oven: 50°C -5°C/min – 180°C
Detector temperature: 280°C
Injector temperature: 160°C
Injection volume: 2 µL
Injection mode: Split 20:1 using SGE Focusliner
Carrier gas: Oxygen-free nitrogen
Flow rate: 1.8 mL/min
Retention time: Geraniol: Approx. 21 min

Sample preparation

Total geraniol content:

Two separate samples are prepared, each in duplicate: 0.7 and 0.8 g test item is weighed into separate 30 mL vials. Internal standard solution IS1, 20 mL is added, the vial is capped, shaken and sonicated to ensure complete dissolution of the sample. Solids are left to settle or, if necessary, the sample is filtered through a GFC filter and quantified by GC-FID. Based on a nominal geraniol content of 6.6%, the sample concentrations are 2.31 and 2.64 mg/mL respectively.

Free geraniol content:

A sufficient amount of test item is transferred to a centrifuge tube and centrifuged at 10 000g for 30 minutes, preferably in a cooled centrifuge. The clear supernatant is collected and subsamples of 1.4 g and 2.0 g (accurately weighed) are transferred to 30 mL vials. 20 mL of internal standard solution IS3 are added to each vial, which are capped and mixed using a roller mixer at 60 rpm for 5 minutes. The layers are allowed to separate and the upper hexane layer is sampled and quantified by GC-FID. Based on a nominal free geraniol content of 0.13%, extract concentrations are 0.091 and 0.13 mg/mL respectively.

Stock solutions and calibration standards

Total geraniol content internal standard solution: 2.5 g of 1-nonanol are transferred to a 1L volumetric flask, dissolved in and adjusted to volume with methanol. This solution is referred to as IS1.

Free geraniol content internal standard solution: 0.5g of 1-nonanol is transferred into a 200 mL volumetric flask, dissolved in and adjusted to volume with hexane. This internal standard is used directly for the preparation of calibration standards. This solution is referred to as IS2.

Free geraniol content internal standard solution for sample analysis: pipette 25 mL of IS2 into a 500 mL volumetric flask and make up to volume with hexane. This solution is referred to as IS3.

Total geraniol standard solutions: for an expected total geraniol content of 6.6% in the test item, analytical grade geraniol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS1. The calibration range spans concentrations from 1 mg/mL to 4 mg/mL. Seven calibration standards are prepared.

Free geraniol standard solutions: for an expected free geraniol content of 0.13% in the test item, analytical grade geraniol (0.02 to 0.08 g) is weighed into a 30 mL vial, dissolved in and diluted with 20 mL Internal standard solution IS2. 1 mL of each solution is diluted into 20 mL hexane, providing a calibration range over the 0.05 mg/mL to 0.2 mg/mL bracket. Six calibration standards are prepared.

Calculations

The same calculations are applied to calculate total geraniol and free geraniol content.

Concentration of geraniol is calculated as follows:

$$\text{A. S. content} = \frac{\text{AE}}{\text{AIS}} \times \frac{\text{Wt IS} \times 100}{\text{Wts} \times \text{FAv.}}$$

Where:

- A.S. content = geraniol content in % w/w
- AE = area geraniol peak in sample
- AIS = area Internal Standard peak in sample
- Wt IS = Weight of Internal Standard solution dilution
- Wts = Weight of sample taken
- FAv. = average Response Factor from the internal standards before and after the sample

To calculate the amount of encapsulated geraniol, the amount of free geraniol is subtracted from the amount of total geraniol.

Accuracy and repeatability

Accuracy was assessed through six independent samples. Technical geraniol is accurately weighed into a 100 mL volumetric flask, in quantities of between $\pm 25\%$ of the nominal geraniol content expected to be found in the formulation. Test item blank formulation is accurately weighed into each flask at a level similar to that used for sample analysis (i.e. between 0.7 and 0.8 g). The contents of the flasks were homogenized carefully and the whole sample is subjected to sample preparation as described above. The final extract concentration is expected in the range of 2.0 to 3.0 mg/mL.

The same technical geraniol is used to prepare the calibration standards used for accuracy and repeatability determination.

Repeatability (precision):

For free geraniol analysis precision, the formulation was centrifuged as described in the sample preparation section; seven replicate samples of the upper layer covering the range of about $\pm 50\%$ of the nominal free geraniol content were weighed out, extracted with hexane and quantified as described above. For total geraniol analysis precision, six replicate samples covering the range of about $\pm 25\%$ of the nominal total geraniol content were weighed out and prepared as described above.

Findings

Linearity:

The method was found to be linear over the range of 1.0 to 4.0 mg/mL. The correlation coefficient was 1.0000 and the equation to the calibration line was $y = 20.957x + 0.0002$. The method is linear.

Specificity:

Samples of test item were prepared without external standard and analysed; no peak was found at the retention time of the internal standard. Samples of blank formulation were prepared as described above and analysed. No peaks were observed at the retention time of geraniol.

No interferences were observed at the retention time of geraniol or the internal standard.

Example chromatograms of samples and calibration standards are presented in the report.

LOD, LOQ:

The LOQ is not required for active substances according to SANCO/3030/99 rev.5. The LOD is not required either.

Accuracy (recovery):

The accuracy of the method was determined at six representing nominal geraniol content $\pm 25\%$. Mean recovery was 99.33% with a relative standard deviation of 0.702%.

Based on Horwitz value of 2.05% for a 60 g/L sample, the Horrat is 0.34.

Repeatability (precision):

Total geraniol: 0.395% (Horwitz = 2.017, Horrat = 0.19)

Free geraniol: 0.781% (Horwitz = 3.667, Horrat = 0.21)

The data indicates that the precision of the method is acceptable.

Conclusions

Total geraniol is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

Free geraniol is determined and quantified in 3AEY after extraction in hexane using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

The method M619 for quantification of geraniol in 3AEY has been demonstrated to be linear, specific, accurate and precise according to SANCO/3030/99 rev.4. In addition, the method also complies with requirements according to SANCO/3030/99 rev.5.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in 3AEY has already been evaluated at EU level. It was performed under GLP according to Guideline SANCO/3030/99 rev.4 and was successfully validated. Validation also complies with SANCO/3030/99 rev.5.

The method is acceptable for the quantification of geraniol in 3AEY.

KCP 5.1.2/02 (A 2.2.1/02 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/02 (A 2.2.1/02 of this dRR)
Report author	Bailey A.
Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues on the surface of grapes by deposit analysis resulting from sequential applications of 3AEY, in Southern Europe (2006)
Report No	AF/11125/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC)
Deviations from current test guideline	Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Geraniol
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°40408
Active substance content:	99.5% w/w (corresponding to 995 g/kg)
Expiry date of lot/batch:	April 2008
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25 m x 0.32 mm internal diameter fused silica capillary coated with PAS-1701 (0.25 µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode,
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Geraniol: Approx. 5.2 min
Monitored ions/transitions:	69 m/z (quantification), 93 and 123 m/z (qualification)

Sample preparation

Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.01, 0.1 and 1.0 mg/kg, extract concentrations are 0.01, 0.1 and 1.0 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen. Procedural recoveries were performed at 0.01 and 0.26 mg/kg, equivalent to 0.01 and 0.26 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for geraniol. Eight calibration standards were quantified. The equation to the calibration line was $1637921x - 11000$ and the correlation coefficient $R = 0.9985$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis of geraniol standard, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol.

No residues at the retention time of geraniol were found in untreated specimens taken from the control plots of each trial and subsequently used for procedural recovery tests.

Copies of relevant chromatograms are provided for standards of geraniol, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol for each ion monitored. Full mass spectrometry scan of geraniol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.01 mg/kg of geraniol residue in sample. The LOD was not stated.

Accuracy, Repeatability (precision):

Fortification level (mg/kg)	Mean recovery (%)	% RSD	n
Validation			
0.01	100	2.9	5
0.5	87	4.9	5
Combined	94	8.3	10
Procedural recoveries			
0.01	96	-	-
0.26	95	-	-
Mean	96	-	-

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability in the matrix was not investigated in this study.

Conclusions

Geraniol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS (three ions monitored).

The method described was acceptably validated according to SANCO/3029/99 rev.4. Full mass spectrometry scan of geraniol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification. Three ions were monitored to confirm analyte identity. The method is acceptable for the quantification of geraniol in grapes. The Limit of Quantification was 0.01 mg/kg for geraniol residues in grape samples.

The method presented herewith is satisfactory and can be applied to quantify geraniol residues in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level. It was performed under GLP, followed Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.
The method is acceptable for the quantification of geraniol residues in grapes.

KCP 5.1.2/03 (A 2.2.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The validation of the method for analysis of geraniol, eugenol and thymol residues in grapes was not previously evaluated at EU level. The method has been validated for the determination of geraniol, eugenol and thymol residues in grapes according to SANCO/3029/99 rev.4. Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD). The limit of quantification (LOQ) of the analytical method was 0.05 mg/kg of geraniol, eugenol and thymol residues in sample. The study has been accepted for the quantification of geraniol, eugenol and residue in grapes.
-------------------	--

Data point:	CP 5.1.2/03 (A 2.2.1/03 of this dRR)
Report author	Cheshire A.
Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern and Northern France, 2007
Report No	AF/12268/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) ENV/JM/MONO(2007)17
Deviations from current test guideline	Although three ions were monitored, validation data is available on one ion only.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, filtered over a 0.45 μm PTFE filter and geraniol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Geraniol
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°70116
Active substance content:	99.0% w/w (corresponding to 990 g/kg)

Expiry date of lot/batch: January 2013
Storage conditions: Ambient

Analysis parameters

Method type: GC-MS
Instrument: Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column: 30 m x 0.25 mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25 µm film)
Oven: 50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature: Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature: 275°C
Injection volume: Not stated
Injection mode: Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas: Helium
Flow rate: 1 mL/min
Ionization mode: Selected ion monitoring (SIM)
Retention time: Geraniol: Approx. 14 min
Monitored ions/transitions: 69 m/z (quantification), 93 and 123 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed.

For samples fortified at 0.05 mg/kg, 0.1 mg/kg, 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg, extract concentrations are 0.025 µg/mL, 0.05 µg/mL, 0.125 µg/mL, 0.25 µg/mL and 0.5 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in ethyl acetate to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/mL)

C = Volume of final extract (mL)

D = Weight of matrix in final volume of extract (g)
E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$
H1 = Peak area of the first bracketing standard solution injection
H2 = Peak area of the second bracketing standard solution injection
F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.0125 – 1.0 µg/mL for geraniol. Seven calibration standards were quantified. The equation to the calibration line was $7234306x - 6586$ and the correlation coefficient $R = 1.0000$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of geraniol, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of geraniol, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest achieved acceptable recovery. LOQ is 0.05 mg/kg of geraniol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL. The LOD was not stated.

Accuracy, Repeatability (precision):

Accuracy was verified at five levels: 0.05, 0.1, 0.25, 0.5 and 1.0 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Geraniol recovery (%)
Procedural recovery	
0.05	101
0.25	98
0.05	100
0.5	95
0.05	104
0.1	103
0.05	107
0.25	100
0.05	105
1.0	96
Mean	101
%RSD	3.9 (n = 10)

In addition, accuracy and precision were also verified at two levels through five independent sample fortifications at 0.05 and 1.0 mg/kg.

Fortification level (mg/kg)	Geraniol recovery (%)	%RSD (n)
validation		
0.05	98	3.2 (n = 5)
1.0	101	1.7 (n = 5)
Overall	99	2.8 (n = 10)

Stability:

Stability of geraniol residue in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Geraniol residues are extracted from raw commodity grapes sample with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

This analytical method for the determination of geraniol residues content in raw commodity grape samples has been acceptably validated by definition of the specificity, the linearity, the accuracy and the

precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled.
The method is confirmed as acceptable for the quantification of geraniol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol residue in grapes was previously evaluated at EU level. It was performed under GLP and complies with SANCO/3029/99 rev.4 guidance.
The method is acceptable for the quantification of geraniol residue in grapes.

KCP 5.1.2/04 (A 2.2.1/04 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/04 (A 2.2.1/04 of this dRR)
Report author	Jones S.
Report year	2012
Report title	Determination of natural background level residues of thymol, eugenol and geraniol in grapes
Report No	S11-03787
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 EU 1999: 1607/VI/97 7032/VI/95 rev.5
Deviations from current test guideline	The method presented is in agreement with Guidance Documents SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4
Previous evaluation	Yes, evaluated and accepted in DAR (Addendum 2012) under data point IIA 6.3; IIIA 8.2
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and geraniol residues are quantified by GC-MS.

The method described herewith follows the validated EAS (formally Agrisearch) method 'Thymol, Eugenol & Geraniol/Crops/DB/08/1'. The method was fully validated in EAS study AF/10728/ED and EAS study AF/12268/ED, which are included in this submission.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Geraniol, Pure
Source and lot/batch no.:	Penta Manufacturing Company, lot n°115223
Active substance content:	98.9% w/w (corresponding to 989 g/kg)
Expiry date of lot/batch:	15 October 2013
Storage conditions:	Refrigerated.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	30 m x 0.25 mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25 µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature:	Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature:	275°C
Injection volume:	Not stated
Injection mode:	Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Geraniol: Approx. 13.4 min
Monitored ions/transitions:	69 m/z (quantification), 93 and 123 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed.

For samples fortified at 0.05, 0.1 and 0.2 mg/kg, extract concentrations are 0.025, 0.05 and 0.1 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.0125 – 1.0 µg/mL of geraniol. Seven calibration standards were quantified. The equation to the calibration line was $6005900x - 25456$ and the correlation coefficient $R = 0.9999$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of geraniol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with geraniol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of geraniol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with geraniol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest achieved acceptable recovery. LOQ is 0.05 mg/kg of geraniol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL. Although the LOD was not stated, the lowest calibration standard concentration (0.0125 µg/mL), equivalent to a theoretical fortification level of 0.025 mg/kg is proposed as LOD.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 0.05, 0.1 and 0.2 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Geraniol recovery (%)
0.05	94
0.2	111
0.05	96
0.1	108
Mean	102
%RSD	8.3

Stability:

Stability of geraniol residues in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Geraniol residues are extracted from raw commodity grapes sample with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. The Limit of Quantification was 0.05 mg/kg for geraniol residues in grape samples. This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of geraniol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission.

The method is acceptable for the quantification of geraniol residues in grapes.

KCP 5.2/03 (A 2.2.1/05 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.2/03 (A 2.2.1/05 of this dRR)
Report author	Brown D.
Report year	2007
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, and 6 months (2006-2007)
Report No	AD/11145/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev.4 SANCO/825/00 rev.7 EU Working Document 7032/VI/95 rev.5
Deviations from current test guideline	The calibration range does not extend to 50% of the LOQ; Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 6.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

The method was validated prior to use and validation data is presented in Agrisearch study AF/11125/ED in the same test facility, which is included in this submission.

Materials and methods

Test material

Test Standards

Name:	Geraniol
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°40408
Active substance content:	99.5% w/w (corresponding to 995 g/kg)
Expiry date of lot/batch:	1 April 2008
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25m x 0.32mm internal diameter fused silica capillary coated with PAS-1701 (0.25µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)

Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Geraniol: Approx. 12.4 min
Monitored ions/transitions:	69m/z (quantification), 93 m/z and 123 (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

Extraction: A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.1 mg/kg, extract concentration is 0.10 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen. Procedural recoveries were performed at 0.10 mg/kg, equivalent to 0.01 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for geraniol. Eight calibration standards were quantified. The equation to the calibration line was $1394106x - 9722$ and the correlation

coefficient $R = 0.9992$ (y = peak area of geraniol, x = concentration of geraniol (in $\mu\text{g/mL}$)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of geraniol, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol.

Interferences or contamination peak were not observed.

Copies of relevant chromatograms are provided for standards of geraniol, untreated grape samples, treated grape samples, untreated grape samples spiked with geraniol for each ion monitored. Full mass spectrometry scan of geraniol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated in study number AF/11125/ED (Bailey, 2008). LOQ is 0.01 mg/kg of geraniol residue in sample. This level equates to a calibration standard concentration of 0.01 $\mu\text{g/mL}$.

Accuracy, Repeatability (precision):

Accuracy was verified at one level of fortification through procedural recoveries.

Fortification level (mg/kg)	0.1
Geraniol recovery (%)	95
%RSD	10
n	8

Stability:

Stability in the matrix was investigated at 1 month, 3 months and 6 months (34, 93 and 185 days).

Results were as follows:

Time point (days)	0	34	95	185
Recovery (%)	99 (n = 3)	41 (n = 3)	21 (n = 3)	29 (n = 3)

Geraniol is not stable for 1 month (34 days) in the matrix.

Conclusions

Geraniol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 μm PTFE filter and quantified by GC-MS(three ions monitored).

The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission. Full mass spectrometry scan of geraniol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification. The Limit of Quantification was 0.01 mg/kg for geraniol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of geraniol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) and SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission.

The method is acceptable for the quantification of geraniol residues in grapes.

KCP 5.2/04 (A 2.2.1/06 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.2/04 (A 2.2.1/06 of this dRR)
Report author	Brown D.
Report year	2012
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, 7, 14 and 28 days, 3, 6 and 12 months after treatment with 3AEY (6.4% w/w geraniol, 3.2% w/w eugenol and 6.4% w/w thymol)
Report No	AD/12351/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) 7032/VI/95 rev.5 SANCO/3029/99 SANCO/825/00
Deviations from current test guideline	The method presented is in line with Guidance Document SANCO/825/00 rev.8.1
Previous evaluation	Partially evaluated (up to 28 days) and accepted in DAR (2011) under data point IIA 6.1.1/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with acetone. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and geraniol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. However due to unavailability of the chromatographic column, a new chromatographic column and oven conditions were used. These changes are not expected to have impact on the analytical method performance except for the retention time of the analytes.

Materials and methods

Test material

Name:	Geraniol
Source and lot/batch no.:	Dr. Ehrenstorfer, lot n°40408 Dr. Ehrenstorfer, lot n°70116
Active substance content:	99.5% (corresponding to 995 g/kg) 99.0% (corresponding to 990 g/kg)
Expiry date of lot/batch:	01 April 2008 22 January 2013
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	2B-Multiresidue-1, 30m x 0.25mm id (0.25µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 40°C/min – 330°C (10 min)

Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume	Not stated
Injection mode	Splitless mode, temperature 250°C
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode	Selected ion monitoring
Retention time:	Geraniol: Approx. 6.8 min
Monitored ions/transitions	69 m/z (qualification), 93 m/z and 123 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and acetone (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 1.0 mg/kg, extract concentration is 0.5 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

Specimen fortification was carried out using a 3AEY prepared in water.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL of geraniol. Five calibration standards were quantified. The equation to the calibration line was $554272x - 786$ and the correlation coefficient $R = 0.9995$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Analysis of untreated control samples indicated a small level of interference from co-extracted material with residues ranging from 1.917 mg/kg at Day 0 and 1.612 mg/kg at Day 1. This was confirmed by the examination of qualification ions where the ion ratios were significantly different to those of standards. However residues were <0.05 mg/kg at Day 3, 7 and 14, but were present at 0.32 mg/kg at Day 28. It was noted that where high residues occurred in control samples, comparable exaggerated values were also observed in treated and procedural recovery samples. The level in the control samples was not consistent across time points but did appear to decrease over time. Significantly lower residues of geraniol were observed in control samples analysed at 14 and 28 days and therefore comparison of results from stored samples versus the nominal applied is considered to be reliable. No residues above the limit of quantitation were determined in untreated control samples stored at 3, 6 or 12 months. In each case for each time point, procedural and treated samples were corrected for the amount found in control samples. Analysis of treated samples showed residues comparable to freshly fortified samples analysed at the same time point. All treated samples showed residues similar to that observed at Day 0, indicating that when geraniol is applied to whole grapes in the diluted 3AEY formulation and subsequently stored frozen, the residues remain stable for 12 months.

Copies of relevant chromatograms are provided for standards of geraniol, untreated grape samples, treated grape samples, untreated and treated grape samples spiked with geraniol for each ion monitored. Full mass spectrometry scan of geraniol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The limit of quantitation is set at 0.05 mg/kg as validated as part of study number AF/10728/ED (Bailey, 2007). This level equates to a calibration standard concentration of 0.025 µg/ml.

Accuracy, Repeatability (precision):

Accuracy was verified at one level of fortification through procedural recoveries at 1.0 mg/kg fortification.

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	103	90	104	100	101	108	90	102	99
Overall	Mean = 99%, RSD = 8.4%; n = 18								

Stability:

Stability of the geraniol residues in the matrix was investigated at 0, 1, 3, 7, 14, 27, 94, 189 and 366 days).

Results were as follows:

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	102 (n = 3)	82 (n = 3)	101 (n = 3)	97 (n = 3)	99 (n = 3)	104 (n = 3)	86 (n = 3)	98 (n = 3)	97 (n = 3)

Geraniol is stable for up to one year in the matrix when stored frozen at -18°C.

Conclusions

Geraniol residues are extracted from raw commodity grapes sample with acetone, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. The Limit of Quantification was 0.05 mg/kg for geraniol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of geraniol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this

submission.

The method is acceptable for the quantification of geraniol residues in grapes.

Avian diet (Ecotoxicology)

KCP 5.1.2/07 (A 2.2.1/07 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in avian diet was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/07 (A 2.2.1/07 of this dRR)
Report author	Martin K.H, Nixon W.B.
Report year	2007
Report title	Analytical method verification for the determination of 3 AEY (thymol/geraniol/eugenol mixture) in avian diet
Report No	648C-101
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and geraniol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Geraniol (reference material)
CAS number:	106-24-1
Source and lot/batch no.:	Sigma-Aldrich, lot n°08107HC
Active substance content:	99.14% w/w (equivalent to 991.4 g/kg)
Expiry date of lot/batch:	Not stated
Storage conditions:	Ambient temperature

Analysis parameters

Method type	GC-FID
Instrument:	Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column:	DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven:	70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume:	2 µL
Injector temperature:	250°C
Injection mode:	Splitless mode
Carrier gas:	Helium, CHP ≈ 28 PSI
Flow rate:	Hydrogen: 40 mL/min Air: 450 mL/min
Retention time:	Geraniol: Approx. 6.7 min

Sample preparation

10 g of fortified diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) to fit within the calibration range:

- 3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 19.68 µg geraniol⁴/mL
- 20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL), equivalent to 13.12 µg geraniol¹/mL

Calibration standards

A geraniol stock solution was prepared by weighing 0.1020 g geraniol analytical standard in a 100 mL volumetric flask and brought to volume with acetone. The concentration of the stock solution was 1010 µg/mL. This stock solution was used to prepare a combined secondary standard solution containing all three substances (eugenol, geraniol and thymol), although only geraniol is considered in this summary. The secondary stock solution was prepared by transferring 10 mL of the primary stock solution into a 100 mL volumetric flask and adjusting to volume using 50:50 (v:v) acetone:hexane. The secondary stock concentration was 101 µg/mL. From this secondary stock solution, calibration standards spanning the concentration range 5.0 to 25.0 µg/mL in acetone/hexane 50:50 (v:v).

Accuracy and Recovery samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 196.8 ppm diet of geraniol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 1443 ppm diet of geraniol)

Calculations

The concentration of geraniol found at the instrument was determined using the following equation:

$$\text{Geraniol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

$$\text{Geraniol (ppm)} = \left(\frac{\text{Geraniol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity}$$

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

⁴ Based on a geraniol content of 6.56% w/w

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 µg/mL. Five calibration standards were quantified. The equation to the calibration line was $33.067x + 0.1645$ and the correlation coefficient $R = 0.9999$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, reagent blank samples, matrix blank samples, and matrix samples spiked with geraniol.

LOD, LOQ:

The Limit of Detection (LOD) was evaluated at the lowest analytical standard analyzed, and was observed to yield a detector response at least five times greater than the peak-to-peak background noise in the matrix blank extracts at the same dilution factor as the lowest matrix fortification.

ppm geraniol equivalent

$$= \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}}$$

The Limit of Quantification (LOQ) was set at 3000 ppm of test item based upon the lowest matrix fortification level analyzed concurrently with the samples, corresponding to 196.8 ppm (196.8 µg/g of diet).

Accuracy, Repeatability (precision):

Diet concentration (ppm)	Geraniol concentration in diet (ppm)	Measured Geraniol in diet (ppm)	%nominal	Mean	SD	RSD
3000	196.8	195	99.1	92.2	6.63	7.19
		177	89.9			
		166	84.3			
		195	99.1			
		174	88.4			
22000	1443	1111	77.0	88.0	7.76	8.82
		1357	94.0			
		1245	86.3			
		1395	96.7			
		1238	85.8			

The data indicates that the accuracy and precision of the method is acceptable at a 196.8 ppm and above in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%. The LOQ for geraniol is therefore set 196.8 ppm (196.8 µg/g of diet).

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and geraniol is quantified by GC-FID. The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in avian diet. The Limit of Quantification 196.8 µg geraniol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in avian diet.

KCP 5.1.2/08 (A 2.2.1/08 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in samples of test diet was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/08 (A 2.2.1/08 of this dRR)
Report author	XXXXXXX
Report year	2007
Report title	3AEY (Thymol/Geraniol/Eugenol Mixture): a dietary LC50 study with the northern bobwhite
Report No	648-102
Document No	Not applicable
Guidelines followed in study	OECD 205 EPA OPPTS 850.2200
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and geraniol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Geraniol (reference material)
Source and lot/batch no.:	Sigma-Aldrich, lot n°08107HC
Active substance content:	99.14% w/w (equivalent to 991.4 g/kg)
Expiry date of lot/batch:	Not stated
Storage conditions:	Ambient temperature

Analysis parameters

Method type	GC-FID
Instrument:	Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column:	DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven:	70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume:	2 µL
Injector temperature:	250°C
Injection mode:	Splitless mode
Carrier gas:	Helium, CHP ≈ 28 PSI
Flow rate:	Hydrogen: 40 mL/min Air: 450 mL/min
Retention time:	Geraniol: Approx. 6.7 min

Sample preparation

Samples of the test diets were collected to verify the test concentrations administered and to confirm the stability and homogeneity of the test substance in the diets.

10 g of diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) depending on their nominal concentration:

- 3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 19.68 µg geraniol⁵/mL
- 5000 ppm: 1/2 (extract concentration: 0.25 mg 3AEY/mL), equivalent to 16.4 µg geraniol⁹/mL
- 10 000 ppm: 1/5 (extract concentration: 0.20 mg 3AEY/mL) equivalent to 13.12 µg geraniol⁹/mL
- 20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL) equivalent to 13.12 µg geraniol⁹/mL

Calibration standards

Calibration standards in the range 5 – 25 µg/mL are prepared in acetone/hexane 50:50 (v/v).

Recovery and precision samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 196.8 ppm diet of geraniol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 1443 ppm diet of geraniol)

Diet homogeneity

Homogeneity of the test substance in the diet was evaluated by collecting six samples from the 5000 and 20000 ppm test diets at the time of preparation on Day 0 and Day 1. Homogeneity samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. The homogeneity samples also served as verification samples for those concentrations.

Calculations

The concentration of geraniol found at the instrument was determined using the following equation:

$$\text{Geraniol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

$$\text{Geraniol (ppm)} = \left(\frac{\text{Geraniol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity}$$

$$\text{ppm geraniol equivalent} = \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}}$$

⁵ Based on a geraniol content of 6.56% w/w

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 µg/mL. Five calibration standards were quantified. The equation to the calibration line was $33.5982x + 0.73655$ and the correlation coefficient $R = 0.9999$ (y = peak area of geraniol, x = concentration of geraniol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, matrix blank samples, and matrix samples spiked with geraniol.

LOD, LOQ:

The Limit of Detection (LOD) was set based upon the injection volume (2.00 µL) and the lowest standard concentration 5.00 µg a.i./mL. The LOD was set at 10.0 ng on-column.

The Limit of Quantification (LOQ) was set at 3000 ppm based upon the lowest matrix fortification level analyzed concurrently with the samples and the LOQ validated during the study 648-101C in the same test facility. The 5.00 µg a.i./mL geraniol standard was equivalent to a calculated value of 50 ppm a.i. in the matrix blank extract. Measured values greater than or equal to the ppm a.i. equivalent were reported for each analyte.

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 3000 ppm (196.8 ppm diet of geraniol) and 22 000 ppm (1443 ppm diet of geraniol).

Fortification level (mg/L)	3000 ppm	22 000 ppm	mean	%RSD	n
Accuracy Day 1	75	69	72	-	2
Accuracy Day 2	83	74	78.5	-	2
Overall			75.3	7.7	4

The method is accurate and precise at a level of 3000 ppm and above.

The results obtained confirmed the accuracy and the precision of the method at 3000 ppm (196.8 ppm diet of geraniol) and above.

Homogeneity:

Diet concentration (ppm)	Geraniol concentration in diet (ppm)		Measured Geraniol in diet (ppm)	%nominal	Mean	SD	RSD
5000	328.0	Top left	327	99.7	79.8	11.0	13.8
		Top right	272	82.9			
		Middle left	231	70.4			
		Middle right	249	75.9			
		Bottom left	229	69.8			
		Bottom right	263	80.2			
20000	1312	Top left	1036	79.0	79.4	1.8	2.2
		Top right	1046	79.7			
		Middle left	1001	76.3			
		Middle right	1039	79.2			
		Bottom left	1068	81.4			
		Bottom right	1058	80.6			

The diet was homogenous.

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and geraniol is quantified by GC-FID. The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission. The results obtained in this study confirmed that the method is linear, accurate and precise and suitable for the quantification of geraniol in avian diet. The Limit of Quantification 196.8 µg geraniol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated as part of study number 648-101C (Martin, Nixon, 2007) at concentration levels relevant to the test's results. The method is acceptable for the quantification of geraniol in avian diet.

Water (Ecotoxicology)

KCA 4.1.2/39 (A 2.2.1/09 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/39 (A 2.2.1/09 of this dRR)
Report author	XXXXXXX
Report year	2008d
Report title	Acute Toxicity of GERANIOL to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test
Report No	34291230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Geraniol 980 (technical grade)
CAS number:	106-24-1
Source and lot/batch no.:	Eden Research plc, lot n°96904
Active substance content:	98.2% w/w (corresponding to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 12.9 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 800 g/L was prepared by dissolving 4000 mg test item into 5.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test fish at the start of the test and just before the test medium renewal.

Test item concentrations were 5.0, 10, 20, 40 and 80 mg/L

Calibration standards: The test item was used to prepare a stock solution. 51.7 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L, corrected for purity.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg dimethylformamide (DMF) /L 50% / 50% to obtain standard solutions in the range from 1.0 to 30 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 2.0, 20 and 100 mg test item/L.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 1.0 – 30 mg/L. Seven calibration standards were quantified. The equation to the calibration line was $106406x - 68696$ and the correlation coefficient $R = 0.9997$ (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, control solvent samples, control water samples, and water samples spiked with geraniol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.077 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 2 mg/L. The mean recovery rate in the fortification level of 2 mg test item/L was 119%. This value is slightly greater than the required range of 70 to 110% and so is considered to be only a minor deviation.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 2, 20 and 100.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
2.0	119*	11	6
20	95	9	6
100	98	15	4
Overall	105	16	16

* The mean recovery rate in the fortification level of 2 mg test item/L was 119%. This value is slightly greater than the required range of 70 to 110% and so is considered to be only a minor deviation.

The method is accurate and precise at a level of 2.0 mg/L and above in that the mean recoveries are just slightly higher than the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 2.0 mg/L.

Stability:

For the determination of the stability of the test item under the test conditions and the maintenance of the

test item concentrations during the test period, samples were taken in duplicate from all old test media and the controls after 48 and 96 hours (the aged test media of the test concentrations of 5.0 and 10 mg/L were 48 hours old). Additionally, at any observation date where all test fish are found to be dead at one test concentration, samples were taken from this concentration (the test media were 24, 2 and 2 hours old at test concentrations of 10, 20 and 40 mg/L). After 48 hours of exposure the mean measured test item concentrations were 78% (71 - 84%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 85% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in fish test medium. The Limit of Quantification was 2.0 mg/L for geraniol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in fish test medium.

KCA 4.1.2/40 (A 2.2.1/10 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/40 (A 2.2.1/10 of this dRR)
Report author	XXXXXX
Report year	2008e
Report title	Acute Toxicity of GERANIOL to Zebra Fish (<i>Danio rerio</i>) in a 96-hour Semi-static Test
Report No	34292230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile or a mixture of acetonitrile /test medium (containing 100 mg DMF/L) 50%/50% if necessary to fit within the calibration range prior to analysis of geraniol content by GC-MS.

Materials and methods

Test material

Name:	Geraniol 980 (technical grade)
CAS number:	106-24-1
Source and lot/batch no.:	Eden Research plc, lot n°96904
Active substance content:	98.2% w/w (equivalent to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 12.9 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile or a mixture of acetonitrile /test medium (containing 100 mg DMF/L) 50% / 50% if necessary, to fit within the calibration range prior to quantification

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 400 g/L was prepared by dissolving 2000 mg test item into 5.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 51 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test fish at the start of the test and just before the test medium renewal.

Test item concentrations were 2.5, 5.0, 10, 20 and 40 mg/L

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg dimethylformamide (DMF) /L 50% / 50% to obtain standard solutions in the range from 1 to 10 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 2, 10 and 40 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter

was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 1 – 10 mg/L. Six calibration standards were quantified. The equation to the calibration line was $87414x - 72146$ and the correlation coefficient $R = 0.9988$ (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, control solvent samples, control water samples, and water samples spiked with geraniol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.1 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 2 mg/L. The mean recovery rate was 113% (n = 4; RSD 12%). However, this is considered not to have any impact on the integrity of the study, as it was only a slight deviation.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 2.0, 10.0 and 40.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
2.0	113*	12	4
10.0	95	6	4
40.0	106	5	4
Overall	105	11	12

* The mean recovery rate in the fortification level of 2 mg test item/L was 113%. This value is slightly greater than the required range of 70 to 110% and so is considered to be only a minor deviation.

The method is accurate and precise at a level of 2.0 mg/L and above in that the mean recoveries are just slightly higher than the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 2.0 mg/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in

the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium. Therefore, results are expressed based on nominal concentrations.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding to the 96 hours test period). Additionally, at any observation date where all test fish were found to be dead at one test concentration, samples were taken from this concentration. After 48 hours of exposure the mean measured test item concentrations were 107% (90 - 127%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 106% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile or a mixture of acetonitrile /test medium (containing 100 mg DMF/L) 50%/50% if necessary to fit within the calibration range prior to analysis of geraniol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in fish test medium. The Limit of Quantification was 2.0 mg/L for geraniol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in fish test medium.

KCA 4.1.2/41 (A 2.2.1/11 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in daphnia test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/41 (A 2.2.1/11 of this dRR)
Report author	Grade R., Wydra V.
Report year	2007a
Report title	Acute Toxicity of GERANIOL to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	34293220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202 EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.3.1.1/01

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Geraniol 980 (technical grade)
CAS number:	106-24-1
Source and lot/batch no.:	Eden Research plc, lot n°96904
Active substance content:	98.2% w/w (corresponding to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 12.8 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 1.2 g test item into 2.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to daphnia. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test organism at the start of the test. Test item concentrations in test media were 3.75, 7.5, 15, 30 and 60 mg/L.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg DMF/L 50% / 50% to obtain standard solutions in the range from 1 to 40 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 3, 15 and 60 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 1 – 40 mg/L. Seven calibration standards were quantified. The equation to the calibration line was $50749x - 58528$ and the correlation coefficient $R = 0.9991$ (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, control solvent samples, control water samples, and water samples spiked with geraniol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.1 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 3 mg/L. The mean recovery rate in the fortification level of 3 mg test item/L was 114%. This value is slightly greater than the required range of 70 to 110% and so is considered to be only a minor deviation as the RSD fulfilled the requirement at this level.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 3.0, 15 and 60 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
3.0	114*	11.0	4
15	96	3.6	4
60	96	1.8	4
Overall	103	11	12

* The mean recovery rate in the fortification level of 3 mg test item/L was 114 %. This value is slightly greater than the required range of 70 to 110 % and so is considered to be only a minor deviation.

The method is accurate and precise at a level of 3.0 mg/L and above in that the mean recoveries are just slightly higher than the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 3.0 mg/L.

Stability:

For the determination of the stability of the test item under the test conditions and the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after 48 hours of exposure) by pouring together the contents of the test beakers of each treatment. Analytical results demonstrate that the test

item was stable in medium. After 48 hours of exposure the mean measured test item concentrations were 100% (86 - 98%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 100% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in daphnia test medium. The Limit of Quantification was 3.0 mg/L for geraniol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in daphnia test medium.

KCA 4.1.2/42 (A 2.2.1/12 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol in daphnia test medium was not previously evaluated at EU level.</p> <p>The method allowing the determination of geraniol in aqueous test medium used for an aquatic toxicity test (Elendt medium M4) has been successfully validated according to the guideline SANCO/3029/99 rev.4.</p> <p>The limit of quantification (LOQ) for geraniol was 0.0120 mg/L in aqueous test medium.</p> <p>The limit of detection (LOD) was 0.0036 mg/L.</p> <p>The mean recoveries were from 70% to 110% at a relative standard deviation (RSD) of $\leq 20\%$.</p> <p>The method is acceptable.</p>
-------------------	---

Data point:	CA 4.1.2/42 (A 2.2.1/12 of this dRR)
Report author	Egeler P
Report year	2021
Report title	Geraniol 98: A Study on the Chronic Toxicity to Daphnia magna [Analytical phase by Shrag K., 2021, Phase ID 20E13110-01-RADW]
Report No	20GC1DB
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev. 4
Deviations from current test guideline	None
Previous evaluation	No, not already evaluated
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are extracted in toluene and quantified by GC-MS.

Materials and methods

Reference item

Test Item

Name:	Geraniol
CAS number:	106-24-1
Source and lot/batch no.:	ECT, Germany (origin: Traditem GmbH), lot n°L4363091
Active substance content:	98.91% w/w (corresponding to 989.1 g/kg)
Expiry date of lot/batch:	31 Dec 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent GC 6890 with MS 5973 detector in EI mode
Analytical column:	Agilent VF-WAXms, 30 m x 0.32 mm i.d., 0.25 µm film thickness; Part No CP 9212
Oven:	100 °C initial, hold 3 min, 40 °C/min to 240 °C, hold 5 min
Injection volume:	2 µL
Injection mode:	Splitless mode
Injection temperature:	240 °C
Carrier gas:	Helium
Flow rate:	1.2 mL/min
Transfer Line temp.:	240 °C
MS Quad temp.:	150 °C
MS Source temp.:	230 °C
Retention time:	Approx. 6.1 min
Monitored ions/transitions	123 m/z, 69 m/z, 111 m/z (30 ms dwell time)

Sample preparation

The test solution specimens were taken out of the freezer storage. Before defrosting, formic acid (1% based on the sample volume; e.g. 0.2 mL to a water sample of 20 mL) and toluene (10:1 (v/v) based on the sample volume; e.g. 2 mL to a water sample of 20 mL) were pipetted into the sample vessels. The samples were then defrosted and homogenised for at least 2 min on a vortex mixer. After phase separation an aliquot of the upper organic phase was transferred into GC vials and used directly for analysis by GC-MS. Final samples were diluted in toluene to achieve final concentrations falling within the range of the calibration curve.

Stock solutions and calibration standards

Stock solution: a stock solution containing 1000 mg/L (S1000) of geraniol was prepared by pipetting 22.8 µL of the reference item into a 20 mL volumetric flask and adjusting the volume to 20 mL with toluene.

Fortification solutions: From this stock solution, fortification solutions of 100 mg/L (S100), 10 mg/L (S10) and 1 mg/L (S1) were prepared in toluene.

Calibration standards: Chromatographic external standard solutions were prepared by diluting the 1 mg/L stock of the reference item with toluene. The calibration range included six standards spanning the range 36 – 1000 µg/L (corresponding to 0.0036 to 0.100 mg/L in the sample).

Fortification samples

Control samples of aqueous test medium were fortified with fortification solutions in order to produce fortified samples at 0.0120 and 1.50 mg/L. Control samples were treated as described for the samples.

Calculations

External standard solutions comparable to the concentration expected in specimens were injected before and after a maximum of 4 samples in the analytical sequence. The concentrations were directly calculated from the peak areas of the samples, using the mean peak area of the two bracketing standards as a one-point-calibration.

The concentration of geraniol in the aqueous test samples in mg/L was calculated as follows:

$$C = \frac{c \times V_{EX} \times d}{V_w \times 1000}$$

Where:

C	Concentration of Geraniol in aqueous test specimens in mg/L
c	Concentration of Geraniol in final sample extracts, corrected with interspersed standards in µg/L
V _{Ex}	Volume of extraction solvent (toluene, 2 mL)
d:	Dilution factor
V _w	Volume of water sample (20 mL)
1000	Conversion factor µg to mg

Recoveries were calculated by the following equation:

$$Rec = \frac{R_{found}}{R_{fortified}} \times 100\%$$

Where:

Rec	Recovery in %
R _{found}	Analyte determined in mg/L
R _{fortified}	Fortification level in mg/L

Findings

Linearity:

Linearity was investigated over the range 36 – 1000 µg/L. Six calibration standards were quantified.

	123 m/z	69 m/z	111 m/z
Equation	y = 56.894 x – 73.66	y = 544.84 x + 607.55	y = 33.500 x + 191.46
R	0.99995	0.99992	0.99991
R ²	0.99989	0.99984	0.99983

The linearity of the method is validated for this range of concentration.

Specificity:

The concentration of geraniol in the samples was determined by gas chromatography with MS detection using at least three characteristic mass fragments. Mass fragment m/z = 123 was used for quantification, mass fragments m/z = 69 and m/z = 111 were used for confirmation. No significant interferences from the test medium were detected at the retention time corresponding to the analyte in any of the control specimens.

Copies of relevant chromatograms are provided in the report for standards of geraniol, control solvent samples, control water samples, and water samples spiked with geraniol.

LOD, LOQ:

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 0.0120 mg/L.

The limit of detection (LOD) was defined as 30% of the limit of quantification. The LOD was found at 0.0036 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 0.0120 and 1.5 mg/L

Fortification level [mg/L]	Recoveries			No of analyses	Overall recovery		Overall recovery	
	Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]	Mean [%]	RSD [%]
Geraniol quantifier mass fragment m/z = 123							98	3.3
0.0120	104, 99, 100, 102, 101	101	1.9	5	98	3.5		
1.50	95, 94, 97, 95, 96	95	1.2	5				
Geraniol qualifier 1 mass fragment m/z = 69								
0.0120	105, 103, 102, 102, 100	102	1.8	5	99	4.0		
1.50	95, 95, 97, 94, 96	95	1.2	5				
Geraniol qualifier 2 mass fragment m/z = 111								
0.0120	98, 97, 94, 99, 95	97	2.1	5	96	1.6		

1.50	97, 95, 96, 95, 96	96	0.9	5				
------	--------------------	----	-----	---	--	--	--	--

Mean recovery values obtained by GC-MS for geraniol for all fortification levels comply with the standard acceptance criteria of guideline SANCO/3029/99 rev. 4, which demands that the mean recovery at each fortification level should be in the range of 70 - 110%. It is therefore concluded, that the method is suitable for aqueous test medium using GC with MS detection.

Moreover, all corresponding relative standard deviations of less than 20% indicate that the method demonstrates good precision and repeatability for aqueous test medium at the validated levels.

Procedural recoveries:

The aqueous test medium samples C2, C3 and C4 (reserve samples according to the study plan) were additionally analysed. Therefore, procedural recoveries, one at the LOQ of 0.0120 mg/L, one at 1.50 mg/L and one control sample were extracted and analysed along with the samples to prove the performance of the method.

Matrix	Fortification Level [mg/L]	Procedural Recovery [%]
Geraniol quantifier mass fragment m/z = 123		
Aqueous test medium (provided by test facility)	0.0120	102
	1.50	98
Geraniol qualifier 1 mass fragment m/z = 69		
Aqueous test medium (provided by test facility)	0.0120	98
	1.50	98
Geraniol qualifier 2 mass fragment m/z = 111		
Aqueous test medium (provided by test facility)	0.0120	101
	1.50	99

With recoveries ranging from 70-110%, the method performance for the day of extraction of the reserve samples is proven.

Stability:

Not all sample extracts were analysed within 24 hours after extraction of geraniol. Therefore, stability testing was performed as follows. The calibration standards in toluene were prepared at the day of extraction and stored refrigerated along with the samples. On the day of analysis, one standard solution in toluene was freshly prepared and analysed against the stored one. If freshly prepared and stored standard solution differed by no more than 20% the stability of the analyte in the final extract is proven.

The stability of geraniol in final extracts under refrigerated conditions for at least 3 days is proven.

Conclusions

Sample of aqueous test medium are extracted in toluene and quantified by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in daphnia test medium. The Limit of Quantification was 0.0120 mg/L mg/L for geraniol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in daphnia test medium was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in daphnia test medium.

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in algal test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/43 (A 2.2.1/13 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008f
Report title	Acute Toxicity of GERANIOL to <i>Pseudokirchneriella subcapitata</i> in an Algal growth Inhibition Test
Report No	34294210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.4/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The samples were diluted with acetonitrile or acetonitrile / test medium (containing 100 mg DMF/L) 50% / 50%, if necessary, to fit within the calibration range prior to analysis by GC-MS.

Materials and methods

Test material

Name:	Geraniol 980
Source and lot/batch no.:	Eden Research plc, lot n°96904
Active substance content:	98.2%
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 12.8 min
Monitored ions/transitions	Not stated

Sample preparation

The samples were diluted with acetonitrile or acetonitrile / test medium (containing 100 mg DMF/L) 50% / 50%, if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 2000 mg test item into 2.0 mL dimethylformamide (DMF). This stock solution was diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added in each test solution. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to algae. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the algae at the start of the test.

Test item concentrations were 1.0, 3.2, 10, 32 and 100 mg/L.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg DMF /L 50% / 50% to obtain standard solutions in the range from 0.5 to 30 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 1, 3, 10 and 100 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$ where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.5 – 30 mg/L. Eight calibration standards were quantified. The equation to the calibration line was $49625x - 37986$ and the correlation coefficient $R = 0.9981$. The method is linear.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

LOD, LOQ:

The Limit of Detection is determined mathematically from the linear calibration curve according to DIN 32 645. The LOD was found at 1.77 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable

mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 3 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 1.0, 3.2, 10, 32 and 100 mg/L

Fortification level (mg/L)	Accuracy ¹	%RSD	n
1.0	< LOQ	Not calculated	4
3.2	87	-	2
10	75	-	2
32	101	-	2
100	96	-	2
Overall	90	12	8

¹: test item concentration was found to be below the LOD in all samples at 96 hours. Results above reflect T₀ sampling times.

Stability:

At test start measured concentrations ranged from 74 % to 102 % of the nominal values. After 96 hours all values were below the Limit of Detection. Under the test conditions the test item was not stable over the test period of 96 hours.

Conclusions

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in algal test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in algal test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in algal test medium.

KCA 4.1.2/44 (A 2.2.1/14 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol in algal test medium was not previously evaluated at EU level.</p> <p>The method has been validated according to the guideline SANCO/3029/99 rev.4.</p> <p>The limit of quantification (LOQ) for geraniol was 0.03 mg test item/L</p> <p>The mean recoveries were from 70% to 110% at a relative standard deviation (RSD) of ≤20%.</p> <p>The validity criteria for the analytical method have been met.</p> <p>The method is acceptable.</p>
-------------------	--

Data point:	CA 4.1.2/44 (A 2.2.1/14 of this dRR)
Report author	Seidel U., Emnet P.
Report year	2021
Report title	Geraniol: Toxicity to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test
Report No	155771210
Document No	Not applicable
Guidelines followed in study	OECD 201, adopted March 23, 2006, corrected July 28, 2011 OECD Series on Testing and Assessment, No. 23, 2nd Ed., February 08, 2019 SANCO/3029/99 rev.4 11/07/00

Deviations from current test guideline None

Previous evaluation No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

The samples were extracted twice with ethyl acetate in the presence of sodium chloride. The organic phase was collected and kept frozen until analysis of geraniol content by GC-MS.

Materials and methods

Test item

Name: Geraniol 98 (pure grade)
CAS number: 106-24-1
Source and lot/batch no.: Traditem GmbH, lot n°L4363091
Active substance content: 98.91% w/w (equivalent to 989.1 g/kg)
Expiry date of lot/batch: December 2021
Storage conditions: Dark, room temperature

Analysis parameters

Method type: GC-MS
Instrument: Agilent GC -7890A, Agilent MSD 5975C, Gerstel Multi Purpose Sampler 2
Analytical column: Agilent VF-WAXms, 30 m x 320 µm x 0.25 µm
Oven: 60 °C hold for 2 min, then 30 °C/min to 240 °C, hold for 1 min
Injection volume: 1.5 µL
Injection mode: Pulsed splitless mode. Injection pulse pressure: 100 kPa
Injection temperature: 240°C
Carrier gas: Helium 5.0
Flow rate: 1.8 mL/min
Transfer line temperature: 250°C
MS ion source: 260°C
MS Quad: 150°C
Acquisition: SIM mode
Retention time: Approx. 6.57 min
Monitored ions/transitions: 69 m/z, 93.1 m/z, 123.1 m/z

Sample preparation

The samples (10 mL) were extracted directly after sampling. Approximately 3.5 g NaCl was added to each sample prior to the start of the extraction. The samples were extracted twice with 10 mL ethyl acetate for 10 minutes on an overhead rotary shaker. The two extracts were combined and stored deep frozen (≤ -20 °C) until analysis. For analysis an aliquot was diluted further with ethyl acetate to match the calibration range, if necessary.

Stock solutions and calibration standards

Stock solution: The test item was used to prepare a stock solution. Approx. 50 mg of the test item were dissolved in 50 mL ethyl acetate (5 minutes ultrasonication) to obtain a stock solution of approximately 1 g test item /L.

Calibration standards: Appropriate amounts of the stock solution were diluted with ethyl acetate to obtain standard solutions in the range from 3 – 300 µg test item/L.

Recovery and precision samples

Approximately 400 mg of the test item were dissolved (5 minutes ultrasonication) in 50 mL ethyl acetate to obtain a stock solution of approximately 8 g test item/L. Two independent stock solutions were prepared. Appropriate amounts of these stock solutions were diluted with ethyl acetate to obtain intermediate solutions of 3 and 300 mg test item/L. Fortification samples of 0.03 and 80 mg test item/L were obtained via a 1:100 dilution step by spiking 9.9 mL of test water with 0.1 mL of the appropriate intermediate solutions.

The samples (10 mL) were shaken well and extracted as described for the biological samples. For analysis an aliquot was diluted further with ethyl acetate to match the calibration range, if necessary

Calculations

Quantification: Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a = slope

b = y-axis intercept

Calculated concentrations: The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

Nominal concentration of fortified samples: For the nominal concentration of fortified samples, the exact weight of the test item used for the preparation of the fortification stock solution needs to be taken into account and is calculated by the equation:

$$C_{\text{nom-corrected}} = C_{\text{NFC}} * ((w/v) / s)$$

Where:

C_{NFC} : nominal fortified concentration

w : weight of test item sample

v : volume of fortification stock solution

s : desired stock solution concentration

Recovery rate: The recovery rate was calculated using the equation

$$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Geometric mean recovery: The geometric mean recovery is calculated as follows.

$$GM = 10^{(\text{term})}$$

Where:

$$\text{term} = \left(\left(\frac{1}{2(t_n - t_1)} \right) \sum_{i=1}^{n-1} [(\log(\text{conc}_i) + \log(\text{conc}_{i+1})) (t_{i+1} - t_i)] \right)$$

Where:

GM: geometric mean recovery [%]

t_1 : initial time < t_2 <... t_n = final time

conc_1 : initial concentration, $\text{conc}_2, \dots, \text{conc}_n$ = final concentration [%]

Findings

Linearity:

Linearity was investigated over the range 3.00 – 300 µg/L. Eleven calibration standards were quantified.

Examples of calibration curves are as follows:

- m/z 69.0 (Quantifier): $y = 647 * x - 1376$ ($r = 0.9997$)
- m/z 93.1 (Qualifier): $y = 140 * x - 21$ ($r = 0.9999$)
- m/z 123.1 (Qualifier): $y = 89 * x + 4$ ($r = 0.9998$)

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided in the report for standards of geraniol, biological control and test samples.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise.

- m/z 69.0 (Quantifier): 0.7 µg test item/L
- m/z 93.1 (Qualifier): 0.9 µg test item/L
- m/z 123.1 (Qualifier): 1 µg test item/L

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 0.03 mg/L:

- m/z 69.0 (Quantifier): 102 % (n = 6, RSD 2 %)
- m/z 93.1 (Qualifier): 98 % (n = 6, RSD 3 %)
- m/z 123.1 (Qualifier): 100 % (n = 6, RSD 2 %)

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 0.03 and 80.0 mg/L

Monitored ion	m/z 69.0		m/z 93.1		m/z 123.1	
Fortification level	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
0.03 mg/L	102%	2 (n = 6)	98%	3 (n = 6)	100%	2 (n = 6)
80.0 mg/L	86%	1 (n = 6)	84%	1 (n = 6)	87%	2 (n = 6)
Overall	94%	10 (n = 12)	91%	8 (n = 12)	93%	8 (n = 12)

The method is accurate and precise at a level of 0.03 mg/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 0.03 mg/L. A confirmatory method is not required, since 3 ions were monitored.

Conclusions

The samples were extracted with ethyl acetate and diluted in ethyl acetate if necessary, to fit within the calibration range prior to analysis of geraniol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in algal test medium. The Limit of Quantification was 0.03 mg/L for geraniol in algal test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in algal test medium was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in algal test medium.

KCP 5.1.2/09 (A 2.2.1/15 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or

for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/09 (A 2.2.1/15 of this dRR)
Report author	XXXXX
Report year	2008a
Report title	Acute Toxicity of 3AEY to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test
Report No	34301230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Geraniol (reference material)
CAS number:	106-24-1
Source and lot/batch no.:	Sigma-Aldrich, lot n°96904
Active substance content:	98.2% w/w (equivalent to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness

Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	not stated
Retention time:	Geraniol: Approx. 12.8 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to geraniol, this is equivalent to 0.41, 0.82, 1.64, 3.28 and 6.56 mg/L respectively, based on a geraniol content of 6.56% w/w in the test item.

Stock solutions and calibration standards

Geraniol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only geraniol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 8 mg geraniol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L and stirred. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 15, 20 and 100 mg test item/L. With respect to geraniol, this is equivalent to 0.33, 0.98, 1.31 and 6.56 mg/L respectively, based on a geraniol content of 6.56% w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.1 – 8 mg/L.

Five calibration standards were quantified. The equations to the calibration line was $279657x - 96311$, the correlation coefficient R was at least 0.9981 (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of geraniol, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.03 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 15 mg test item/L, corresponding to 0.983 mg geraniol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 5.0, 15.0, 20.0 and 100 mg/L, corresponding to 0.33, 0.98, 1.31 and 6.56 mg geraniol/L respectively.

Test item fortification level (mg/L)	Accuracy	%RSD	n
5	224*	16.3	4
15	110	3.1	4
20	96	5.9	4
100	98	10.3	6
Overall	101	9	14*

Recoveries at 5 mg test item/L (0.33 mg geraniol/L) were not acceptable and therefore were excluded from the calculations. The LOQ is therefore set at 15 mg test item /L (0.98 mg geraniol/L).

The data indicates that the accuracy and precision of the method is acceptable at a 15.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 15.0 mg test item/L or 0.98 mg geraniol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding to the 96 hours test period). After 48 hours of exposure the mean measured test item concentrations were 67% (61 - 75%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 82% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in fish test medium.

The Limit of Quantification was 0.98 mg geraniol/L in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in fish test medium.

KCP 5.1.2/10 (A 2.2.1/16 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in daphnia test medium was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/10 (A 2.2.1/16 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008b
Report title	Acute Toxicity of 3AEY to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	34302220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202 EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Geraniol (reference material)
CAS number:	106-24-1
Source and lot/batch no.:	Sigma-Aldrich, lot n°96904
Active substance content:	98.2% w/w (equivalent to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector

Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	Not stated
Retention time:	Geraniol: Approx. 12.8 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to geraniol, this is equivalent to 0.41, 0.82, 1.64, 3.28 and 6.56 mg/L respectively, based on a geraniol content of 6.56% w/w in the test item.

Stock solutions and calibration standards

Geraniol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only geraniol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 8 mg geraniol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L and stirred. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 10, 20 and 100 mg test item/L. With respect to geraniol, this is equivalent to 0.33, 0.66, 1.31 and 6.56 mg/L respectively, based on a geraniol content of 6.56 % w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.1 – 8 mg/L.

Eight calibration standards were quantified. The equation to the calibration line was $y = 222858x - 44329$, the correlation coefficient R was 0.9982 (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for geraniol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.06 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 20 mg test item/L, corresponding to 1.31 mg geraniol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 5.0, 20.0 and 100 mg/L, corresponding to 0.33, 1.31 and 6.56 mg geraniol/L respectively,

Test item fortification level (mg/L)	Accuracy	%RSD	n
5	175*	13.8	4
20	92	5.1	4
100	99	6.7	4
Overall	97	5	8

*Accuracy was not acceptable at 5 mg/L. These values were not included in the overall calculations.

The data indicates that the accuracy and precision of the method is acceptable at a 20.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 20.0 mg test item/L or 1.31 mg geraniol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after the end of 48 hours test period). After 48 hours of exposure the mean measured test item concentrations were 75% (67 - 79%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 83% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in daphnia test medium. The Limit of Quantification was 1.31 mg geraniol/L in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in daphnia test medium.

KCP 5.1.2/11 (A 2.2.1/17 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of geraniol in alga test medium was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/11 (A 2.2.1/17 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008c
Report title	Toxicity of 3AEY to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test
Report No	34303210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Geraniol
CAS number:	106-24-1
Source and lot/batch no.:	Sigma-Aldrich, lot n°96904
Active substance content:	98.2% w/w (equivalent to 982 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min –

	240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	Not stated
Retention time:	Geraniol: Approx. 12.9 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 1.0, 3.2, 10.0, 32.0 and 100 mg/L. With respect to geraniol, this is equivalent to 0.066, 0.21, 0.66, 2.10 and 6.56 mg/L respectively, based on a geraniol content of 6.56% w/w.

Stock solutions and calibration standards

Geraniol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only geraniol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.07 to 5 mg geraniol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 1, 10 and 100 mg test item/L. With respect to geraniol, this is equivalent to 0.066, 0.66, and 6.56 mg/L respectively, based on a geraniol content of 6.56% w/w.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.07 – 5 mg/L through two separate concentration ranges: Low level range = 0.07 – 1.0 mg/L and high level range = 0.5 – 5 mg/L.

Six calibration standards were quantified. The equation to the calibration line was $y = 182144x - 12416$, the correlation coefficient R was 0.9958 (y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified

reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for geraniol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.04 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 10 mg test item/L, corresponding to 0.66 mg geraniol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 1.0, 10.0 and 100 mg/L, corresponding to 0.066, 0.66 and 6.56 mg geraniol/L respectively,

Test item fortification level (mg/L)	Accuracy	%RSD	n
1	Not calculated – below LOQ		4
10	94	22.9 ¹	4
100	98	1.6	4
Overall	96	14.9	8

¹ The relative standard deviation of the fortification level of 10 mg test item/L was 23% in case of Geraniol. This is only slightly higher than the required maximum value of 20% and is considered not to have an impact on the integrity of the study, as it was only a slight deviation.

The data indicates that the accuracy and precision of the method is acceptable at a 1.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is slightly higher of the limit value of 20% but is considered not to have an impact on the integrity of the study. The LOQ is therefore set at 10.0 mg test item/L or 0.656 mg geraniol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls (containing algae) were taken at the end of the test (after the 96 hours test period). Under the test conditions geraniol was not stable. All reported results in the report amendment are expressed in terms of the geometric mean concentrations of the test item.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of geraniol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of geraniol in alga test medium. The Limit of Quantification was 0.656 mg geraniol/L in alga test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in alga test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in alga test medium.

KCP 5.1.2/12 (A 2.2.1/18 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The validation of the method for analysis of geraniol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level. The HPLC-UV/DAD analytical method for the determination of eugenol, geraniol and
-------------------	--

	<p>thymol in water stock solution and in sugar feeding solution treated with test item ARAW was fully validated, according to SANCO/3029/99 rev.4 guidance document.</p> <p>The Limit of Quantification of geraniol was 40.9 mg/kg in sugar feeding solution and 196.5 mg/L in water stock solution.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD).</p> <p>The study is acceptable.</p>
--	--

Data point:	CP 5.1.2/12 (A 2.2.1/18 of this dRR)
Report author	Aversa S
Report year	2019
Report title	Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document)
Report No	BT081/19
Document No	Not applicable
Guidelines followed in study	SANCO 3029/99/rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Water stock solution

Sample of aqueous stock solution are diluted in acetonitrile to fit within the calibration range prior to analysis by HPLC-DAD.

50% w/v sugar feeding solution

The 50% w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test item

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L) Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L) Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)
Product density:	1.027 kg/L
Expiry date of lot/batch:	October 2020
Storage conditions:	Ambient, dark.

Analytical standard

Name:	Geraniol
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV6051
Active substance content:	99.4% w/w (corresponding to 994 g/kg)
Expiry date of lot/batch:	July 2020
Storage conditions:	2 – 8°C, dark.

Origin of samples

The method was used to analyse the water stock solution coming from the ecotoxicological study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure, Pecorari F., 2019) and the sugar feeding solution coming from the ecotoxicological study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees (*Apis mellifera* L.), 10-day feeding laboratory test, Pecorari F., 2019).

Analysis parameters

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile
Gradient:	Isocratic 60% A/ 40% B
Flow rate:	0.6 mL/min
Retention time:	Geraniol: Approx. 2.2 min
Detector wavelength:	210 nm

Sample preparation

Water stock solution

The water stock solutions originate from study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure, Pecorari F., 2019).

The nominal concentration of the test water stock solutions is 100 g product/L at high level and 6.25 g product/L at low level.

The high-level stock solution is diluted with acetonitrile, first 0.4 mL in 10 mL to produce a 4.0 g product/L solution, then 1 mL in 10 mL to produce a final sample of concentration 0.40 g product/L.

The low-level stock solution is diluted with acetonitrile 0.6 mL in 10 mL to produce a final sample of concentration 0.375 g product/L. Geraniol concentration in the diluted stocks is presented below:

ARAW content in dilution (g/L)	Geraniol content in ARAW	Geraniol content in dilution (mg)	Geraniol extract concentration (mg/L)
0.40	6.46% w/v or 62.90 mg/g (density 1.027)	25.16	25.16
0.375		23.58	23.58

Sugar feeding solution

The test sugar feeding solution originates from study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees (*Apis mellifera* L.), 10-day feeding laboratory test, Pecorari F., 2019).

The nominal concentration of the sugar feeding solution is 50000 mg product/kg (equivalent to 50 mg product/g - high level) and 1300 mg product/kg (equivalent to 1.3 mg product/g - low level).

Samples of test feeding solution (1 g) are mixed with water (2 mL) and acetonitrile (10 mL) and sonicated. Phase separation is obtained by the addition of sodium chloride, shaking and settling. The acetonitrile phase is collected and filtered. At that point, extract concentration is 0.13 mg product/mL low level and 5 mg product/mL high level. The low-level extract is analysed directly, while the high-level extract is further diluted (1 mL in 10 mL) to produce an extract concentration of 0.5 mg product/mL prior to analysis by HPLC-DAD.

High- and low-level extract geraniol concentrations are presented below:

ARAW content in extract (g/L)	Geraniol content in ARAW	Geraniol content in dilution (mg)	Geraniol extract concentration (mg/L)
0.13	6.46% w/v or 62.90 mg/g (density 1.027)	8.177	8.177
0.50		31.45	31.45

Blank feeding solutions are subjected to the same procedure to produce a blank matrix extract used for dilution of standards for matrix effect investigation.

Stock solutions and calibration standards

105.4 mg of geraniol analytical standard were weighted in a 10 mL volumetric flask, dissolved and made up to volume with methanol. This Stock Solution was named SS1Ger and had a geraniol concentration of 10476.76 mg/L.

2 mL of this stock was transferred to a 10 mL volumetric flask and diluted to volume with acetonitrile to produce a 2095.3520 mg/L stock (identification: SS2 mix⁶).

Linearity standards – water stock solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (geraniol 209.5352 mg/L). From this stock, five linearity standards spanning the range 3 – 73 mg/L were prepared in acetonitrile.

Linearity standards – sugar feeding solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (geraniol 209.5352 mg/L). From this stock, five linearity standards spanning the range 1 – 73 mg/L were prepared in acetonitrile.

Recovery and precision samples

Water stock solution

Recovery and precision samples are prepared at two levels in water: 120 000 mg product /L and 3125 mg product /L. These solutions were diluted in acetonitrile to obtain the following concentrations:

High-level accuracy: 1 mL in 10 mL followed by 0.25 mL in 10 mL to afford a final concentration of 0.3 g product/L.

Low-level accuracy: 1 mL in 10 mL to afford a final concentration of 0.313 g product/L.

High- and low-level accuracy samples geraniol concentrations are presented below:

ARAW content in extract (g/L)	Geraniol content in ARAW	Geraniol content in dilution (mg)	Geraniol extract concentration (mg/L)
0.3	6.46% w/v or 62.90 mg/g (density 1.027)	18.87	18.87
0.3125		19.66	19.66

Sugar feeding solution

A 6329.19 mg product/L stock solution is prepared in water to use as fortification solution for the sugar feeding solution.

Blank sugar feeding solution is fortified levels using the aforementioned stock solution in water to produce fortified samples at 60000 mg product/kg (equivalent to 60 mg product/g - high level) and 650 mg product/kg (equivalent to 0.65 mg product/g - low level). Fortified sugar feeding solutions are extracted as described above to produce extract of concentrations 0.065 g product/L (low-level) and 6.0 mg product/L (high level). The low-level extract is analysed directly while the high-level is further dilute 1 mL in 10 mL with acetonitrile.

High- and low-level accuracy samples geraniol concentrations are presented below:

ARAW content in extract (g/L)	Geraniol content in ARAW	Geraniol content in dilution (mg)	Geraniol extract concentration (mg/L)
0.065	6.57% w/v or 63.97 mg/g (density 1.027)	4.16	4.16
0.60		38.38	38.38

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

⁶ This SS2 mix stock also contains the other two analytes, eugenol and thymol, which are not considered in this assessment and are treated separately.

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation: % of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Matrix effects:

Matrix effect were investigated by preparing two calibration standards either in matrix (water or sugar feeding solution extract) or in acetonitrile and comparing response for suppression or enhancement. No significant (> 20%) suppression nor enhancement was observed in any of the matrices and calibration standards were prepared in acetonitrile.

Specificity:

The specificity for the three analytes was established by the comparison of the UV Spectrum of the standard solution and a high-level recovery solution. In addition, specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of active substances (eugenol, thymol and geraniol), blank test medium samples (water and sugar feeding), and matrix samples spiked with actives substances (eugenol, thymol and geraniol).

	Water stock solution		Sugar feeding solution	
Linearity	3 – 73 mg/L, n = 5 Y = 7.9278x – 1.4104 R = 1.0000		1 – 73 mg/L, n = 5 Y = 7.9086x – 1.1304 R = 1.0000	
Accuracy	120000 mg prod./L Eq. to 7548 mg geraniol/L	3125 mg prod./L Eq. to 196.5 mg geraniol/L	60000 mg prod./kg Eq. to 3774 mg geraniol/kg	650 mg prod./kg Eq. to 40.89 mg geraniol/kg
	105.18%	100.45%	107.21%	104.04%
%RSD	2.48%	1.77%	1.00%	2.31%
LOQ	3125 mg prod./L Eq. to 196.5 mg geraniol/L		650 mg prod./kg Eq. to 40.89 mg geraniol/kg	
LOD	3 mg/L (lowest calibration level)		1 mg/L (lowest calibration level)	

The method is accurate and precise for geraniol at a level of 40.9 mg/kg and above in sugar feeding solution and at 196.5 mg/L in water stock solution.

The data indicates that the accuracy and precision of the method is acceptable for the fortification level in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability was not investigated.

Conclusions

Geraniol is extracted from test medium (sugar feeding solution and water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of geraniol content is linear, accurate and precise and suitable for the quantification of geraniol in aqueous stock solution and sugar feeding solution. The Limit of Quantification was 40.9 mg/kg in sugar feeding solution and 196.5 mg/L in water stock solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in aqueous stock solution and sugar feeding solution.

KCP 5.1.2/14 (A 2.2.1/19 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol in aqueous stock solutions from bee larvae chronic studies was not previously evaluated at EU level.</p> <p>The method for quantification of geraniol in aqueous stock solution used in the 22-day larval toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19).</p> <p>The LOQ of geraniol was 196.5 mg/L in water stock solution.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.1.2/14 (A 2.2.1/19 of this dRR)
Report author	Pecorari F.
Report year	2019b
Report title	Effects of ARAW on honeybees (<i>Apis mellifera</i> L.) 22-day larval toxicity test with repeated exposure
Report No	BT060/19
Document No	Not applicable
Guidelines followed in study	OECD Guidance Document, No. 239 "Honey Bee (<i>Apis mellifera</i> L.) Larval Toxicity test, Repeated Exposure (15-Jul-2016)".
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous stock solution are diluted in acetonitrile to fit within the calibration range prior to analysis by HPLC-DAD.

Although the study report refers to ARAW (alternative name of 3AEY) which contains eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L) Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L) Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)

Product density: 1.027 kg/L
Expiry date of lot/batch: October 2020
Storage conditions: Ambient, dark.
Analytical standard
Name: Geraniol
Source and lot/batch no.: Sigma Aldrich, lot n°BCBV6051
Active substance content: >99.0%
Expiry date of lot/batch: July 2020
Storage conditions: 2-8°C, dark.

Analysis parameters

Method type: HPLC-DAD
Instrument: Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column: Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature: 30°C
Injection volume: 2 µL
Eluent A: Water with 0.1% trifluoroacetic acid
Eluant B: Acetonitrile
Gradient: Isocratic 60% A/ 40% B
Flow rate: 0.6 mL/min
Retention time: Geraniol: Approx. 2.2 min
Detector wavelength: 210 nm

Method validation

The method for quantification of thymol in aqueous stock solution used in the 22-day larval toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Geraniol is extracted from test medium (water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of geraniol content is linear, accurate and precise and suitable for the quantification of geraniol in aqueous stock solution. The Limit of Quantification was 196.5 mg/L in water stock solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in aqueous stock solutions from bee larvae chronic studies was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in aqueous stock solutions.

Sugar feeding solution (Ecotoxicology)

KCP 5.1.2/13 (A 2.2.1/20 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level.</p> <p>The concentrations of the active substances eugenol, geraniol and thymol in feeding solutions were analyzed.</p> <p>The analysis of samples was performed following the analytical method validated in a dedicated GLP study BT081/19, in compliance with the guideline SANCO/3029/99 rev. 4.</p> <p>The LOQ of geraniol was 40.89 mg/kg in sugar feeding solution.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/13 (A 2.2.1/20 of this dRR)
Report author	Pecorari F.
Report year	2019a
Report title	Chronic oral effects of ARAW on adult worker honeybees <i>Apis mellifera</i> L., 10-day feeding laboratory test
Report No	BT059/19
Document No	Not applicable
Guidelines followed in study	OECD Guideline for the testing on chemicals 245 “Honey bee (<i>Apis mellifera</i> L.), Chronic Oral Toxicity test (10-day feeding test in the laboratory)”.
Deviations from current test guideline	None.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The 50 % w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to ARAW (alternative name of 3AEY) which contains eugenol, geraniol and thymol, only geraniol is considered in this summary.

Materials and methods

Test material

Name:	ARAW
Source and lot/batch no.:	Eden Research plc, lot n°BT-89
Active substance content:	Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L) Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L) Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)
Product density:	1.027 kg/L
Expiry date of lot/batch:	October 2020
Storage conditions:	Ambient, dark.

Analytical standard

Name:	Geraniol
Source and lot/batch no.:	Sigma Aldrich, lot n°BCBV6051
Active substance content:	>99.0%
Expiry date of lot/batch:	July 2020
Storage conditions:	2-8°C, dark.

Analysis parameters

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with

	Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile
Gradient:	Isocratic 60% A/ 40% B
Flow rate:	0.6 mL/min
Retention time:	Geraniol: Approx. 2.2 min
Detector wavelength:	210 nm

Method validation

The method for quantification of geraniol in aqueous stock solution used in the 10-day chronic adult toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Geraniol is extracted from test medium (sugar feeding solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of geraniol content is linear, accurate and precise and suitable for the quantification of geraniol in sugar feeding solution. The Limit of Quantification was 40.89 mg/kg in sugar feeding solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of geraniol in sugar feeding solutions.

Water, buffer solutions,... (Properties)

KCA 4.1.2/47 (A 2.2.1/21 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol in aqueous and n-octanol solutions was not evaluated at EU level.</p> <p>The n-octanol/water partition coefficient of the test item was determined according to OECD guideline 107 and EC method A.8.</p> <p>The test was performed at 3 different ratios of n-octanol and water buffered at 3 different pH values (pH 4, 7 and 9). The determined value of the log of the partition coefficient was within the acceptable range of ± 0.3 log units.</p> <p>The contents of geraniol in aqueous and n-octanol solutions were determined by HPLC with UV detection.</p> <p>The analytical method was validated following SANCO/3029/99, rev.4 with regard to linearity of detector response, precision, accuracy and non-analyte interference of the analytical system.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ (RSD).</p>
-------------------	---

	The limit of quantification (LOQ) for geraniol in aqueous phase was 0.25 mg/L. The limit of quantification (LOQ) for geraniol in n-octanol phase was 0.45 mg/L. The study is acceptable.
--	--

Data point:	CA 4.1.2/47 (A 2.2.1/21 of this dRR)
Report author	Lingott J
Report year	2020
Report title	Partition coefficient of Geraniol (Shake-Flask Method)
Report No	S20-06645
Document No	Not applicable
Guidelines followed in study	EEC A8, OECD 107
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The contents of geraniol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

Test material

Analytical grade of geraniol was used as test item.

Test material

Test Item

Name:	Geraniol, technical grade
CAS number:	106-24-1
Source and lot/batch no.:	Dr. Ehrenstorfer, lot G986607,
Substance content:	97.00% w/w (equivalent to 970 g/kg)
Expiry date of lot/batch:	10.10.2022
Storage conditions:	-18°C, dark.

Analysis parameters

Method type	HPLC-UV
Instrument:	Agilent 1260 Infinity
Analytical column:	Kinetex ® 2.6 µm C18 100A, 150 x 4,6 mm, 2,6 µm, Phenomenex
Column temperature:	40°C
Detector wavelength	194 nm, bandwidth: 2 nm
Injection volume	10 µL
Eluent A:	Acetonitrile
Eluent B:	Ultra-pure water containing 0.1% phosphoric acid
Flow rate:	1 mL/min
Gradient	

Time [min]	% A	% B
0.0	50	50
5.0	50	50
5.5	90	10
8.5	90	10
9.0	50	50
13.0	50	50

Retention time: Geraniol: Approx. 4.4 min

Sample preparation

pH 4, pH 7 and pH 9 buffers are prepared and saturated in n-octanol prior to the test. Correspondingly, samples of n-octanol are saturated with individual buffer solutions.

Stock solutions of concentration 1000 mg/L are individually prepared in buffer-saturated octanol. These stocks are combined with the corresponding octanol-saturated pH 4, pH 7 or pH 9 buffer and equilibrated as part of the test. The concentration of the test item was determined in both phases. To minimize the risk of including traces of n-octanol into the aqueous phases, the n-octanol phase was removed completely before sampling the aqueous phase.

Prior to analysis, the aqueous phase was diluted by a factor of two with acetonitrile, while the octanol phase was diluted by a factor of 20 with acetonitrile and by a factor of 50 with 1/1 acetonitrile/ultra-pure water v/v (total dilution factor: 1000). Diluted samples were quantified by HPLC-UV.

Calibration standards preparation

A stock solution containing 1 mg/mL was prepared in acetonitrile. This stock was sequentially diluted in acetonitrile to produce analytical standards within the range 0.1 – 10 mg/L.

Procedural recovery samples

Procedural recovery samples were prepared either in n-octanol or in a specifically prepared buffer mix.

Aqueous phase, low recovery

2.5 mL of a low spike solution containing 0.5030 mg/L geraniol in acetonitrile were transferred into a 5.0 mL volumetric flask and adjusted to volume with buffer mix.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of geraniol in the aqueous phase is 0.2515 mg/L.

Aqueous phase, high recovery

2.5 mL of a high spike solution containing 1.509 mg/L geraniol in acetonitrile were transferred into a 5.0 mL volumetric flask and adjusted to volume with buffer mix.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of geraniol in the aqueous phase is 0.75 mg/L.

n-Octanol phase, low recovery

To 1 mL of a low spike solution containing 452.7 mg/L geraniol in acetonitrile 1 mL n-octanol were added. The samples were filled up to a volume of 20 mL with acetonitrile. In a second dilution step 0.5 mL of this solution was filled up to a final volume of 25 mL with acetonitrile/ultra-pure water 1/1 v/v.

This corresponds to a final dilution factor of 1000. The sample was directly used for analysis. Five samples were prepared. The nominal content of geraniol in the n-octanol phase is 0.45 mg/L.

n-Octanol phase, high recovery

1 mL of a high spike solution containing 1509 mg/L geraniol in acetonitrile were transferred into a vial and 1 mL n-octanol were added. The samples were filled up to a volume of 20 mL with acetonitrile. In a second dilution step 0.5 mL of this solution was filled up to a final volume of 25 mL with acetonitrile/ultra-pure water 1/1 v/v.

This corresponds to a final dilution factor of 1000. The sample was directly used for analysis. Five samples were prepared. The nominal content of geraniol in the n-octanol phase is 1.5 mg/L.

Calculations

The concentration of analyte in the measured sample was calculated by the following equations:

$$C = \left(\frac{(A - b)}{a} \right) \cdot d \cdot \frac{v_{std}}{v_s}$$

With

$A = a \cdot C + b$ (calibration equation)

A = response ['Area']

b = Y-axis intercept of the calibration curve ['Area']

a = slope of the calibration curve ['Area']/(mg/L)]

C = concentration of analyte in measuring sample [mg/L]

d = dilution factor

v_{std} = injection volume of standards

v_s = injection volume of sample

The amount of analyte in the measuring sample was calculated by the following equations:

$$M = \frac{C \cdot V}{1000 \text{ mL/L}}$$

with

M = amount of analyte in measuring sample [mg]

V = volume of the aqueous or n-octanol phase [mL]

The calibration curve was determined from standards of analyte which were measured parallel to the samples.

Findings

Linearity: Linearity was verified within the concentration bracket 0.1 to 2 mg/L at pH 4 and 7, and within the concentration bracket 0.2 to 2 mg/L at pH 9, through the quantification of eight standards. The equations to the calibration line were respectively:

- at pH 4: $6.523632x + 0.003897$ and the correlation coefficient $R = 1.0000$
- at pH 7: $6.507185x + 0.015858$ and the correlation coefficient $R = 1.0000$
- at pH 9: $6.532545x - 0.013210$ and the correlation coefficient $R = 1.0000$

(y = peak area of geraniol, x = concentration of geraniol (in mg/L)).

The linearity of the method is validated for this range of concentration at different pH.

Specificity:

Blank samples of the buffers and n-octanol were prepared as described above and quantified by HPLC-UV. No signal was observed in any of the blank samples at the retention time of geraniol. No interferences were observed.

Copies of relevant chromatograms are provided for standards of geraniol, blank samples containing buffer mix, blank samples containing n-octanol, aqueous phase of sample with buffer pH 4, 7 and 9, n-octanol phase of sample with buffer pH 4, 7 and 9, and recovery samples for n-octanol phase and for aqueous phase.

LOD, LOQ:

The limit of quantification (LOQ) for geraniol in aqueous phase was 0.2515 mg/L (lowest recovery concentration).

The limit of quantification (LOQ) for geraniol in n-octanol phase was 0.45 mg/L (lowest recovery concentration).

The limit of detection (LOD) for geraniol was 0.1 mg/L (nominal concentration of the lowest calibration standard) for pH 4 and 7, and 0.2 mg/L for pH 9.

Accuracy, Repeatability (precision):

Phase	Recovery level (mg/L)	Recovery (%)	%RSD	Horrat value	n
Aqueous	0.503	99.9	1.8	0.15	5
	1.509	100.8	1.3	0.13	5
Overall		100.4	1.6	-	10
n-Octanol	452.7	98.8	0.6	0.14	5
	1509	97.5	0.6	0.17	5
Overall		98.2	0.9	-	10

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (80 -120%) and the Horrat values obtained are well below the limit value of 1.

Conclusions

The contents of geraniol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

The method for quantification of geraniol in solutions from water/n-octanol partition coefficient testing was fully validated for specificity, linearity, accuracy and precision. The method is acceptable for the quantification of geraniol in water/n-octanol partition coefficient testing solutions.

Assessment and conclusion by applicant:

The validation of the method for analysis of geraniol in aqueous and n-octanol solutions was performed under GLP according to Guideline SANCO/3030/99 rev.4 and was successfully validated. Validation also complies with SANCO/3030/99 rev.5.
The method is acceptable for the quantification of geraniol in physicochemical testing solutions.

A 2.2.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.2.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

GRAPES

KCP 5.2.1/05 (A 2.2.2.1/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method was not not previously evaluated at EU level.</p> <p>Grape bunches samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANCO/3029/99, rev.4 for grapes matrix in the EAS Study S20-06528.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in grapes is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations, except for geraniol and thymol where the blank value was around 50%.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.1.2/05 (A 2.2.2.1/01 of this dRR)
Report author	Chadwick G
Report year	2021a
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to grapevine, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F, 2021, report n°S20-06337-L1]
Report No	S20-06337
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol is quantified after extraction from grape samples, by GC-MS (three ions monitored).
The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical standard

Name:	Geraniol
CAS No.:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, lot n°SHBL2152
Active substance content:	99.1%
Expiry date of lot/batch:	August 2021
Storage conditions:	Ambient, dark.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column:	Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven:	100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature:	280°C
Injection volume:	1 µL
Injector temperature:	250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas:	Helium
Flow rate:	1.1 mL/min
Ionisation mode	Electron Impact Ionisation (EI)
Acquisition type:	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Retention time:	Geraniol: approx. 11.5 min
Monitored ions (m/z)	Geraniol: 123 (30 ms dwell) for quantification, 69 (30 ms dwell) and 41 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step.

For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of geraniol: the geraniol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of

recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of grape were fortified prior to extraction with the fortification solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control grape samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the geraniol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 123) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of geraniol residues in grape and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $115x + 3002$, the correlation coefficient R was 0.9992 (y = peak area of geraniol, x = concentration of geraniol (in ng/mL)).

The linearity of the method is validated for this range of concentration of geraniol.

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

Specificity:

Specificity was studied by analysis samples of standards of geraniol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with geraniol, treated grape sample.

Interference or contamination peak was detected in control samples at 40% LOQ; it is assumed this interference at the same retention time of geraniol is caused by the natural presence of geraniol in grapes, as documented in published articles presented in the residue section. In addition, validation was performed using matrix-matched standard. Despite the natural presence of geraniol in grapes, the method is considered specific.

Copies of relevant chromatograms are provided for standards of geraniol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with geraniol, treated grape sample for the quantification ions (m/z 123).

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in grape.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the grapes for the quantification ions (m/z 123).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	88	22*	4
0.10	97	8	6
Overall	93	14	10

*It is believed the precision value was affected by the level of interference observed, presumed to be due to the natural geraniol concentration in grapes. However, the overall precision is acceptable and the data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

Conclusions

Geraniol is quantified after extraction from grape samples, by GC-MS (three ions monitored).

This analytical method for the determination of geraniol content in grapes samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4

requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in grapes. The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol residues in grapes was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated.

The method is acceptable for the quantification of geraniol in grapes.

KCP 5.2/01 (A 2.2.2.1/02 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in grapes was not previously evaluated at EU level.</p> <p>The analytical method has been fully validated for the determination of eugenol, methyl-eugenol, thymol and geraniol in grapes samples according to the SANTE/2020/12830, rev.1. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification was 0.01 mg/kg for eugenol, methyl-eugenol, thymol and geraniol in grapes.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The studies are acceptable.</p>
-------------------	--

Data point:	CP 5.2/01 (A 2.2.2.1/02 of this dRR)
Report author	Driss F
Report year	2021a
Report title	Validation of Residue Method for the Determination of Eugenol, Geraniol, Thymol and Methyl-Eugenol in Grape
Report No	S20-06528
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830, rev.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method was used in Eurofins studies S20-06337 and S20-06526 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which are included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only geraniol is

considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number SHBL2152
Substance content:	99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch:	19 August 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow	50 mL/min at 0.05 min
Injection volume	1 µL (depending on sensitivity)
Injector temperature	250°C
Column oven temperature programme	

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:	Approx. 11.5 min for geraniol
Ionisation mode	Electron Impact Ionisation (EI)
Scan type	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Solvent delay	6 min
Ions monitored	Geraniol: 123 # (100 ms dwell), 69 (100 ms dwell), 41 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

An amount of 10 g ± 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL

polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of grapes which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of geraniol in initial grapes samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of grapes on the GC-MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
$C_{\text{Solv-Std}}$	Nominal concentration of standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 103 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 7 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 2 %, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C _A	Concentration of analyte in final extract (ng/mL)
A _A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A _{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = A _A – (mean) blank peak area
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali1} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions: m/z 123, 69 and 41.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Geraniol (%)		
		Quantification (m/z 123)	Confirmation (m/z 69)	Confirmation (m/z 41)
Grapes	80	(+) 6.0	(-) 4.9	(-) 10.7

Matrix suppression or enhancement was $\leq 20\%$ in grapes for geraniol thus deemed to be insignificant. However, it was shown to be significant for thymol (see below section for thymol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for each ion:

- m/z 123: $y = 78x + 3783$; $r = 0.9979$, $r^2 = 0.9958$
- m/z 69: $y = 670x + 1276$; $r = 0.9972$, $r^2 = 0.9944$
- m/z 41: $y = 377x - 567$; $r = 0.9974$, $r^2 = 0.9949$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.002 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in grape matrix.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 123 (Proposed for Quantification)							
Grapes	0.01	99; 116; 101; 103; 110	106	7	5	108	7
	0.1	114; 122; 105; 105; 109	111	6	5		
Fragment m/z 69 (Proposed for Confirmation)							
Grapes	0.01	102; 116; 95; 93; 105	102	9	5	107	9
	0.1	115; 122; 109; 106; 109	112	6	5		
Fragment m/z 41 (Proposed for Confirmation)							
Grapes	0.01	106; 119; 98; 97; 114	107	9	5	109	8
	0.1	114; 121; 109; 102; 108	111	6	5		

Observable peak was detected in control sample extract (interference around 60% of LOQ). Recoveries are corrected for the mean peak areas of the control sample extracts.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of geraniol, control matrix, and control matrix spiked with geraniol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the geraniol.

A blank correction was performed with the mean peak area of the control sample(s) used for fortification for the three ions monitored for geraniol (interference around 60% of LOQ). Recoveries and matrix-matched standards were corrected by background subtraction. In addition, three ions were monitored and quantified. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix, and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ions monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10% of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 103 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Geraniol was found to be stable in final extracts of grape for 7 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Geraniol - Fragment m/z 123 (Proposed for Quantification) [#]						
Grape	0	0.01	99; 116; 101; 103; 110	106	7	(-) 19
	7		69; 91; 82; 75; 111	86	19	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Observable peak was detected in control sample extract (interference around 60 % of LOQ). Recoveries are corrected for the mean peak areas of the control sample extracts.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Geraniol is extracted from grape matrix using acetonitrile and solid-phase extraction clean-up, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity. This analytical method for the determination of geraniol content in grape matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in grapes. The method presented herewith is satisfactory and was applied to quantify geraniol in grapes in Eurofins study S20-06337 and S20-06526 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in grapes was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1 and was successfully validated.

The method is acceptable for the quantification of geraniol in grapes.

KCP 5.2/02 (A 2.2.2.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The study “ <i>Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions</i> ” was not previously evaluated at EU level. Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06528. Quantification was performed by use of GC-MS detection. The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte. The study is acceptable for the quantification of geraniol in grapes.
-------------------	---

Data point:	CP 5.2/02 (A 2.2.2.1/03 of this dRR)
Report author	Driss F.
Report year	2021b
Report title	Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions
Report No	S20-06526
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the

acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within study n°S20-06528 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number SHBL2152
Substance content:	99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch:	19 August 2021
Storage conditions:	Ambient

Analysis parameters

Method type

GC-MS

Instrument:

Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)

Analytical column:

Carrier Gas

Helium (constant pressure / constant flow: 1.1 mL/min)

Injection mode

Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz

Purge Flow

50 mL/min at 0.05 min

Injection volume

1 µL (depending on sensitivity)

Injector temperature

250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:

Approx. 11.5 min for geraniol

Ionisation mode

Electron Impact Ionisation (EI)

Scan type

SIM (Selected Ion Monitoring)

Ion source temperature

230°C

Quadrupole temperature

150°C

Solvent delay

6 min

Ions monitored

Geraniol: 123 # (100 ms dwell)

Since the method was already validated for specificity, this study only monitored a single ion.

Sample preparation

An amount of 10 g ± 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of grapes which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of geraniol in initial grapes samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by single extraction and single injection.

Since the method had already been validated, matrix effects, LOQ was not investigated, and a single ion was monitored.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries ($R_{ProcRec}$) are calculated as follows:

$R_{Rel} (\%)$ and $R_{ProcRec} (\%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{Corrected}$) is calculated as follows:

$R_{Corrected} (\%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0

analysis ($P_{\text{Remaining}}$) is calculated as follows:

$R_{\text{Remaining}} (\%) =$	$\frac{R_{\text{Rel_Mean}}}{R_{\text{Day0_Mean}}} \times 100$
$R_{\text{Rel_Mean}}$	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{\text{Day0_Mean}}$	Unrounded mean R_{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Geraniol	m/z 123: $y = 85x + 4565$; $r = 0.9987$, $r^2 = 0.9975$
----------	---

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.002 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0 storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The following recoveries were obtained:

Matrix	Fortification level (mg/kg)		Recovery (%)					Mean (%)	Rel. Std. Dev. (%)
		0 days	43 days	111 days	154 days	280 days			
	Geraniol								
Grapes	0.1	100, 97, 101	96	100	110	109	102	5	

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06528 for the quantification of residues of geraniol in grape matrix was used to assess the stability of residues of geraniol in grapes. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify geraniol in grapes.

Assessment and conclusion by applicant:

The method used to verify the stability of geraniol residue in grapes was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06528 and verified as part of this study. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1. The method is acceptable for the quantification of geraniol in grapes.

APPLES

KCP 5.1.2/06 (A 2.2.2.1/04 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol residues in apples was not previously evaluated at EU level.</p> <p>Apple fruit samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANCO/3029/99, rev.4 for apple matrix in the EAS Study S20-06529.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in apple is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/06 (A 2.2.2.1/04 of this dRR)
Report author	Chadwick G.
Report year	2021b
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to apple, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F., 2021, report n° S20-06361-L1]
Report No	S20-06361
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol is quantified after extraction from apple samples, by GC-MS (three ions monitored). The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission. Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical standard

Name: Geraniol
CAS No.: 106-24-1
Source and lot/batch no.: Sigma Aldrich, lot n°SHBL2152
Active substance content: 99.1%
Expiry date of lot/batch: August 2021
Storage conditions: Ambient, dark.

Analysis parameters

Method type: GC-MS
Instrument: Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column: Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven: 100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature: 280°C
Injection volume: 1 µL
Injector temperature: 250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas: Helium
Flow rate: 1.1 mL/min
Ionisation mode: Electron Impact Ionisation (EI)
Acquisition type: SIM (Selected Ion Monitoring)
Ion source temperature: 230°C
Quadrupole temperature: 150°C
Retention time: Geraniol: approx. 10.0 min
Monitored ions (m/z): Geraniol: 123 (30 ms dwell) for quantification, 69 (30 ms dwell) and 41 (30 ms dwell) for confirmation

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step. For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of geraniol: the geraniol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract. The same calibration range was also prepared in solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of apple were fortified prior to extraction with the fortification solutions. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control apple samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{end} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the geraniol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 123) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of geraniol residues in apple and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $25x + 701$, the correlation coefficient R was at least 0.9992 (y = peak area of geraniol, x = concentration of geraniol (in ng/mL)).

The linearity of the method is validated for this range of concentration of geraniol.

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

Specificity:

Specificity was studied by analysis samples of standards of geraniol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with geraniol, treated apple sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of geraniol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of geraniol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with geraniol, treated apple sample for the quantification ions (m/z 123).

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in apple.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the apples for the quantification ions (m/z 123).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	87	17	8
0.10	82	8	8
Overall	85	14	16

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 69 and 41).

Conclusions

Geraniol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

This analytical method for the determination of geraniol content in apples samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in apples.

The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol residues in apples was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was

successfully validated.
The method is acceptable for the quantification of geraniol in apples.

KCP 5.2/05 (A 2.2.2.1/05 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in apples was not previously evaluated at EU level.</p> <p>The method was found to be valid according to the guidance document SANTE/2020/12830, rev.1 for the determination of eugenol, geraniol, thymol and methyl-eugenol in apples with the tested LOQ of 0.01 mg/kg.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for three (3) selected MS fragment ions for eugenol, geraniol, thymol and methyl-eugenol are within 70 – 110% with relative standard deviations \leq 20% and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.</p> <p>The LOQ was successfully established at 0.01 mg/kg in apples for all three ions.</p> <p>The LOD was set at the level of the lowest acceptable calibration standard which is 0.0025 mg/kg.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/05 (A 2.2.2.1/05 of this dRR)
Report author	Driss F
Report year	2021c
Report title	Validation of residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in apple
Report No	S20-06529
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method was used in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1

Source and lot/batch no.: Sigma Aldrich, batch number SHBL2152
Substance content: 99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch: 19 August 2021
Storage conditions: Ambient

Analysis parameters

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C
Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 11.5 min for geraniol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Geraniol: 123 # (30 ms dwell), 69 (30 ms dwell), 41 (30 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

An amount of 10 g ± 0.1 g of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution. Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of geraniol in initial apple samples). All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of apples on the GC-MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
$C_{\text{Solv-Std}}$	Nominal concentration of standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 103 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 15 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 2 %, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of three ions: m/z 123, 69 and 41.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Geraniol (%)		
		Quantification (m/z 123)	Confirmation (m/z 69)	Confirmation (m/z 41)
Apples (NEU)	80	(+) 11.1	(+) 4.3	(+) 82.5
Apples (SEU)	80	(-) 8.2	(-) 12.7	(-) 4.9

Matrix suppression or enhancement was $\geq 20\%$ in apples for geraniol thus deemed to be significant. Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.002 mg/kg to 0.4 mg/kg in initial Apple samples. Linearity was confirmed for each ion:

- m/z 123: $y = 12x + 6$; $r = 0.9976$, $r^2 = 0.9953$
- m/z 69: $y = 97x + 723$; $r = 0.9979$, $r^2 = 0.9958$
- m/z 41: $y = 59x + 676$; $r = 0.9981$, $r^2 = 0.9963$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in apple matrix.

50 samples at LOQ and 50 samples at 10xLOQ were tested for apples because some background residues in the controls that might be quite variable were expected.

Geraniol							
Matrix	Fortificati on Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)

Fragment <i>m/z</i> 123 (Proposed for Quantification)							
Apples	0.01	96, 93, 103, 117, 114, 95, 89, 119, 138, 116, 123, 109, 123, 112, 107, 93, 78, 108, 112, 102, 105, 118, 133, 89, 99, 98, 105, 85, 98, 84, 90, 91, 92, 76, 79, 90, 71, 77, 79, 81, 76, 84, 77, 95, 92, 78, 74, 82, 70, 76	96	18	50	92	19
	0.1	112, 96, 117, 120, 112, 102, 108, 85, 100, 89, 109, 99, 109, 98, 103, 97, 106, 102, 94, 89, 92, 106, 110, 112, 67, 69, 75, 67, 69, 85, 67, 74, 75, 76, 77, 72, 60, 72, 70, 73, 90, 90, 76, 58, 81, 80, 71, 70, 79	88	19	49 #		
Fragment <i>m/z</i> 69 (Proposed for Confirmation)							
Apples	0.01	89, 84, 95, 105, 110, 83, 78, 114, 136, 117, 113, 101, 119, 99, 105, 89, 72, 107, 106, 95, 101, 106, 140, 83, 101, 98, 109, 75, 101, 85, 93, 99, 100, 72, 78, 86, 71, 74, 72, 84, 76, 95, 84, 105, 95, 83, 80, 86, 76, 72	94	18	50	91	19
	0.1	113, 97, 119, 122, 113, 103, 110, 87, 101, 91, 112, 100, 108, 99, 105, 97, 107, 102, 94, 90, 94, 106, 111, 114, 65, 67, 77, 73, 67, 82, 70, 75, 75, 75, 75, 73, 60, 71, 69, 75, 86, 89, 76, 56, 79, 79, 73, 68, 80	88	20	49 #		
Fragment <i>m/z</i> 41 (Proposed for Confirmation)							
Apples	0.01	92, 86, 95, 110, 112, 89, 80, 119, 144, 119, 115, 104, 117, 106, 105, 91, 72, 109, 108, 104, 105, 118, 144, 81, 99, 101, 112, 86, 100, 82, 92, 101, 104, 80, 81, 87, 69, 76, 78, 84, 77, 81, 79, 99, 96, 75, 77, 90, 73, 77	96	18	50	90	20
	0.1	109, 92, 113, 116, 108, 97, 104, 83, 97, 85, 106, 96, 101, 94, 99, 94, 101, 97, 90, 86, 88, 102, 106, 107, 64, 66, 73, 65, 65, 79, 65, 72, 73, 73, 75, 70, 58, 68, 67, 71, 85, 87, 74, 54, 79, 78, 70, 65, 78	85	19	49 #		

Dixon test was used to exclude one value out of the range (around 600%)

No observable peak was detected in any control sample extract. Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of geraniol, control matrix, and control matrix spiked with geraniol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the geraniol.

Blank correction was not performed. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix, and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ions monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 103 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Geraniol was found to be stable in final extracts of apple for 15 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 25) (%)	Rel. Std. Dev. (n = 25) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Geraniol - Fragment m/z 123 (Proposed for Quantification)						
Apple	0	0.01	98, 105, 85, 98, 84, 90, 91, 92, 76, 79, 90, 71, 77, 79, 81, 76, 84, 77, 95, 92, 78, 74, 82, 70, 76	84	11	(-) 15
	15		86, 72, 89, 69, 72, 73, 83, 71, 60, 60, 74, 70, 70, 74, 74, 72, 70, 76, 69, 72, 62, 60, 66, 62, 73	71	10	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Geraniol is extracted from apple matrix using acetonitrile and solid-phase extraction clean-up, then quantified by GC-MS using three separate ions for quantification and confirmation of method specificity. This analytical method for the determination of geraniol content in apple matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in apples. The method presented herewith is satisfactory and was applied to quantify geraniol in apples in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in apples was not previously evaluated at EU level. It was performed under GLP according to Guideline SANTE/2020/12830 rev.1 and was successfully validated.

The method is acceptable for the quantification of geraniol in apples.

KCP 5.2/06 (A 2.2.2.1/06 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	The study “Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions” was not previously evaluated at EU level. Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06529. Quantification was performed by use of GC-MS detection. The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte.
-------------------	---

	<p>The recoveries for each of the analytes at each interval were within 70 – 110%, except at 30 days and 287 days, where the values were in the range 70-123%. The overall mean recoveries were in the range 70-110% and the deviations at 30 days and 287 days were considered not to affect the integrity of the results.</p> <p>The overall mean relative standard deviation covering all testing intervals was $\leq 20\%$ for all analytes.</p> <p>With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the storage samples.</p> <p>The study is acceptable.</p>
--	---

Data point:	CP 5.2/06 (A 2.2.2.1/06 of this dRR)
Report author	Driss F.
Report year	2021d
Report title	Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions
Report No	S20-06527
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within study n°S20-06529 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number SHBL2152
Substance content:	99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch:	19 August 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 μ m film thickness,

Carrier Gas Thames Restek)
Injection mode Helium (constant pressure / constant flow: 1.1 mL/min)
Purge Flow Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz
Injection volume 50 mL/min at 0.05 min
Injector temperature 1 µL (depending on sensitivity)
Column oven temperature 250°C
programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 11.5 min for geraniol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Geraniol: 123 m/z
Since the method was already validated for specificity, this study only monitored a single ion.

Sample preparation

An amount of 10 g ± 0.1 g of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube is then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of geraniol in initial apple samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by single extraction and single injection.

Since the method had already been validated, matrix effects were not investigated, and a single ion was monitored per analyte.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries ($R_{ProcRec}$) are calculated as follows:

$R_{Rel} (\%)$ and $R_{ProcRec} (\%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{Corrected}$) is calculated as follows:

$R_{Corrected} (\%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0 analysis ($P_{Remaining}$) is calculated as follows:

$R_{Remaining} (\%) =$	$\frac{R_{Rel_Mean}}{R_{Day0_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
R_{Day0_Mean}	Unrounded mean R_{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial apple samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Geraniol	m/z 123: $y = 79x + 174$; $r = 0.9982$, $r^2 = 0.9963$
----------	--

The method is linear over the calibration range.

LOQ, LOD:

The LOQ of the method was verified as part of Study n°S20-06529 (Driss 2021c). the LOQ of the method is 0.01 mg/kg.

The LOD of the method was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0

storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The following recoveries were obtained:

Matrix	Fortification level (mg/kg)			Recovery (%)				Mean (%)	Rel. Std. Dev. (%)
		0 days	30 days	112 days	161 days	285 days	287 days		
	Geraniol								
Apples	0.1	87, 67, 87	117	102	99	105	121 *	98	18

* Reanalysis performed to confirm results obtained at 285 days

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06529 for the quantification of residues of geraniol in apple matrix was used to assess the stability of residues of geraniol in apples. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify geraniol in apples.

Assessment and conclusion by applicant:

The method used to verify the stability of geraniol residue in apples was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06529 and verified as part of this study. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1.

The method is acceptable for the quantification of geraniol in apples.

TOMATO, CUCUMBER AND STRAWBERRY

KCA 4.2/01 (A 2.2.2.1/07 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the methods for analysis for geraniol in tomato, cucumber and strawberry was not previously evaluated at EU level.</p> <p>The analytical method was been validated for the determination of geraniol and thymol in tomato, cucumber and strawberry with the tested LOQ of 0.05 mg/kg according to the guidance documents SANCO/825/00 rev. 8. 1 and SANCO/3029/99.</p> <p>LOQ of 0.05 mg/kg was established for geraniol and thymol in tomato, cucumber and strawberry.</p> <p>For tomato and strawberry, five recovery determinations each were performed at the levels of 0.05 mg/kg (LOQ) and 0.50 mg/kg, representing full validation sets according to the guidance documents SANCO/825/00 rev. 8.1 and SANCO/3029/99.</p> <p>For cucumber, only a reduced validation set with each three recovery determinations at the levels of 0.05 mg/kg (LOQ) and 0.50 mg/kg was performed, since cucumber belongs to the same crop group as tomato(= high water) and therefore a reduced validation is sufficient according to SANCO/3029/99.</p> <p>The mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CA 4.2/01 (A 2.2.2.1/07 of this dRR)
Report author	Wiesner F., Breyer N.
Report year	2017
Report title	Validation of an Analytical Method for the Determination of Residues of Geraniol and Thymol in Tomato, Cucumber and Strawberry
Report No	S16-03357
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol is quantified in tomatoes, cucumber and strawberries using LC-MS/MS and a multi-residue method based on QuEChERS⁷. Various extraction techniques were used that are described below.

Although the study report refers to thymol and geraniol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item

Test material

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	LGC Labor GmbH, 86199
Active substance content:	99.0% w/w (equivalent to 990 g/kg)

⁷ EN 1 5662:2008 "Foods of plant origin - Determination of pesticide residues using GC-MS and/or LCMS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE – QuEChERS Method"; German version (2009).

Expiry date of lot/batch: 26 May 2018
Storage conditions: $\leq -18^{\circ}\text{C}$, dark

Test system

Tomato (fruit, high water)
Cucumber (fruit, high water)
Strawberry (fruit, high acid)

Analysis parameters

Method type

Instrument:

Analytical column:

Column temperature:

Injection volume

Mobile phase:

Gradient:

Flow rate:

Ionisation type:

Polarity:

Scan type:

Retention time:

Monitored ions/transitions

Strawberries

LC-MS/MS

1290 Binary Rapid Resolution LC System, Agilent Technologies

API 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Luna Phenyl-Hexyl, 100 mm x 4.6 mm, 3 μm , Phenomenex
60°C

40 μL

Eluant A: Acetonitrile; Eluent B: Water + 5 mM ammonium acetate

Time (min)	Eluent A	Eluent B
1.0	50	50
1.0 – 6.0	50 \rightarrow 90	50 \rightarrow 10
6.0 – 6.1	90 \rightarrow 95	10 \rightarrow 5
6.1 – 7.0	95	5
7.0 – 7.1	95 \rightarrow 50	5 \rightarrow 50
7.1 – 10	50	50

0.6 mL/min

ESI, Turbolon spray

Negative

MS/MS, Multiple Reaction Monitoring (MRM)

approx. 4.3 min

Geraniol: 172 \rightarrow 81 (quantification)

172 \rightarrow 79 (confirmation)

Tomatoes and cucumbers

LC-MS/MS

1290 Binary Rapid Resolution LC System, Agilent Technologies

API 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Luna Phenyl-Hexyl, 100 mm x 4.6 mm, 3 μm , Phenomenex
30°C

40 μL

Eluant A: Acetonitrile; Eluent B: Water + 5 mM ammonium acetate

Time (min)	Eluent A	Eluent B
0.00 – 3.00	25 \rightarrow 95	75 \rightarrow 5
3.0 – 6.00	95	5
6.00 – 6.01	95 \rightarrow 25	5 \rightarrow 75
6.01 – 9.00	25	75

Flow rate:

0.6 mL/min

Ionisation type:

ESI, Turbolon spray

Polarity:

Negative

Scan type:

MS/MS, Multiple Reaction Monitoring (MRM)

Retention time:

approx. 4.3 min

Monitored ions/transitions

Geraniol: 172 \rightarrow 81 (quantification)

172 → 79 (confirmation)

Sample preparation

Where necessary, samples are fortified prior to extraction. The solvent is allowed to evaporate before starting the extraction procedure.

Extraction and liquid/liquid partition:

Homogenized specimen (10 g) are weighed into 50 mL Sarsedt centrifuge tubes. 10 mL of acetonitrile (V_{EX}) is added to the tube which is capped and shaken for 15 minutes using a platform shaker. Then 4 g magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate are added, the centrifuge tube is capped and immediately shaken by hand or using a vortex for about 2 minutes. After shaking, the sample tube is centrifuged for 5 minutes at 4000 rpm.

SPE clean-up:

6 mL of the upper acetonitrile phase are diluted with 54 mL ultra-pure water. The SPE cartridge is conditioned using 1 x 5 mL acetonitrile and 1 x 5 mL ultra-pure water. The sample solution (60 mL) is transferred to the cartridge and allowed to percolate through under gravity. The eluate is discarded. A test tube is placed under the cartridge and 6 mL of acetonitrile are passed through the cartridge without vacuum.

For tomatoes and cucumbers, 800 μ L of the eluate are mixed with 200 μ L ultra-pure water, mixed well and transferred to vials for quantification by LC-MS/MS.

For strawberries, 800 μ L of the eluate are mixed with 200 μ L of 0.5% acetic acid, mixed well and transferred to vials for quantification by LC-MS/MS.

Stock solutions, calibration standards, fortification solutions

A stock solution of concentration 400 μ g/mL was prepared by accurately weighing 10.0 mg or test item into a 25 mL volumetric flask, dissolving and adjusting to volume with acetone.

A fortification solution of concentration 50 μ g/mL is prepared from the stock solution by dilution in acetonitrile. An aliquot of this solution is further diluted in acetonitrile to produce a second fortification solution of concentration 5.0 μ g/mL.

Calibration standards in acetonitrile: water (80:20 v/v) are prepared by sequential dilution of the stock solution in acetone. The calibration range covers the concentration range 12 – 6000 ng/mL and includes 10 standards.

Calibration standards were also prepared in acetonitrile: 0.5% acetic acid (80:20 v/v). They were prepared by sequential dilution of the stock solution in acetone and span the concentration range 12 – 6000 ng/mL. This calibration range includes 10 standards.

In addition, matrix-matched standards are prepared in strawberry extract. They are produced by dilution of standards in acetonitrile: water (80:20 v/v) using blank strawberry extracts, span the concentration range 12 – 600 ng/mL and include 7 standards.

Investigation of matrix effects showed no matrix effect was observed in cucumber and tomatoes. Therefore, solvent standards were used in these matrices.

At least 7 standards are used for each calibration.

Accuracy (recovery) samples

Recovery samples are prepared at two fortification levels, 0.05 mg/kg and 0.5 mg/kg in each matrix (strawberry, cucumber and tomato). Samples are homogenized, fortified and the solvent is allowed to evaporate prior to extraction. Samples are then prepared according to the procedure described under Sample preparation above.

Following extraction and clean-up, a 0.05 mg/kg fortified sample yield an extract of concentration 40 ng/mL, while a 0.5 mg/kg fortified sample yields a 400 ng/mL extract.

Calculations

The evaluation of the results was based on the average response factor which was calculated from the calibration standards. At least 7 external standard solutions were used for the determination of the average response factor.

The residues (R) in mg/kg were calculated according to the following equation:

$$R = \frac{A_A \times V_{Ex} \times V_{End}}{AvF \times G \times V_{Ali} \times CF} \times DF$$

Where:

R = Residues of the analyte in mg/kg

A_A = Peak area of the analyte in the sample solution in counts

AvF = Average response factor

The average response factor is calculated as follows:

$$AvF = \frac{A_{St1}/C_{St1} + A_{St2}/C_{St2} + \dots + A_{StN}/C_{StN}}{N}$$

C_{st} = Concentration of external standard solution in ng/mL

A_{st} = Peak area in the external standard solution in counts

N = Number of external standard solutions

V_{Ex} = Extraction volume: 10 mL

V_{Au} = Aliquot volume: 0.80 mL

V_{End} = Final volume: 1.0 mL

G = Sample weight of the analytical specimen: 10 g

CF = Conversion factor for ng into µg (= 1000)

DF = Dilution factor (1 = no dilution)

Percent recovery from fortified specimen was calculated using the following expressions:

$$Recovery (\%) = \frac{R_{fortified}}{F} \times 100$$

Where:

R_{fortified} = Residues of fortified specimen, in mg/kg

F = Fortification, in mg/kg

Findings

Linearity:

Tomato and Cucumber 172 → 81 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 9951.6144x – 46235.3165 Coefficient of determination R ² : 0.9992 Coefficient of correlation r: 0.9996
	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 9063.3459x – 15825.3542 Coefficient of determination R ² : 1.0000 Coefficient of correlation r: 1.0000
Tomato and Cucumber 172 → 79 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 1855.5802x – 2938.2281 Coefficient of determination R ² : 0.9997 Coefficient of correlation r: 0.9998
Strawberry 172 → 81 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 552.6799x + 1151.8769 Coefficient of determination R ² : 0.9985 Coefficient of correlation r: 0.9992
	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 577.0570x + 3041.1061 Coefficient of determination R ² : 0.9992 Coefficient of correlation r: 0.9996
Strawberry 172 → 79 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 97.5028x + 17.0233 Coefficient of determination R ² : 0.9968 Coefficient of correlation r: 0.9984

Specificity:

LC-MS/MS determination was conducted by monitoring two mass transitions for geraniol (172 → 81 m/z and 172 → 79 m/z). Due to enhanced sensitivity mass transition 172 → 81 m/z is proposed to be used for quantification, but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two control samples for tomato and strawberry were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For the reduced validation set of cucumber, one control only was extracted

and analysed. For both mass transitions, the samples showed no significant interference (above 30 % of LOQ) at the retention time of the analyte in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Copies of relevant chromatograms for both geraniol mass transition (172 → 81 m/z and 172→ 79 m/z) are provided for solvent standard solution, untreated specimen of each matrices (strawberry, cucumber and tomato), specimen spiked with geraniol of each matrices (strawberry, cucumber and tomato).

Moreover, MS/MS spectrum (initial product ion scan) of Geraniol ion [M+H]⁺ (172 m/z) are provided and confirmed the selected mass transition 172 → 81 m/z and 172→ 79 m/z for the analyte.

LOD, LOQ:

The LOQ of the method is defined as the lowest analyte concentration at which the methodology had been successfully validated. Although considerable efforts were made to meet the recommended LOQ of 0.01 mg/kg, this was not achievable taking into account the available instrumentation and the nature of the analytes. Therefore, during this validation an LOQ of 0.05 mg/kg was established for geraniol in tomato, cucumber and strawberry.

The LOD was set at 30% of the LOQ, which is 0.015 mg/kg. Peaks at the LOD were equivalent to or more than three times the background noise.

Accuracy, Repeatability (precision):

Crop, transition	Fortification	Mean Recovery	% RSD
Tomato 172 → 81 m/z	0.05 mg/kg	88%	7.7%, n = 5
	0.5 mg/kg	101%	4.5%, n = 5
	Combined	94%	9.4%, n = 10
Tomato 172 → 81 m/z	0.05 mg/kg	100%	7.5%, n = 5
	0.5 mg/kg	107%	2.3%, n = 5
	Combined	103%	6.2%, n = 10
Tomato 172 → 79 m/z	0.05 mg/kg	87%	4.1%, n = 5
	0.5 mg/kg	92%	4.2%, n = 5
	Combined	90%	4.9%, n = 10
Cucumber 172 → 81 m/z	0.05 mg/kg	75%	7.3%, n = 5 ³
	0.5 mg/kg	92%	5.6%, n = 5 ³
	Combined	84%	12.5%, n = 10 ⁶
Cucumber 172 → 79 m/z	0.05 mg/kg	75%	6.1%, n = 5 ³
	0.5 mg/kg	85%	5.8%, n = 5 ³
	Combined	80%	8.6%, n = 10 ⁶
Strawberry 172 → 81 m/z	0.05 mg/kg	110%	2.4%, n = 5
	0.5 mg/kg	110%	1.7%, n = 5
	Combined	110%	2.0%, n = 10
Strawberry 172 → 81 m/z	0.05 mg/kg	101%	3.7%, n = 5
	0.5 mg/kg	97%	2.7%, n = 5
	Combined	99%	3.6%, n = 10
Strawberry 172 → 79 m/z	0.05 mg/kg	109%	5.5%, n = 5
	0.5 mg/kg	110%	4.4%, n = 5
	Combined	110%	4.7%, n = 10

The data indicates that the accuracy and precision of the method is acceptable for all test matrices (tomato, cucumber and strawberry) for both geraniol mass transition (172 → 81 m/z and 172→ 79 m/z), in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Solvent calibration solutions were found to be stable for 19 days when stored at 1 – 10°C in the dark.

Extracts were found to be stable for at least 16 days for tomato and 10 days for strawberry when stored at

1°C to 10°C in the dark.

Matrix effects:

Matrix effects were observed during the analysis of geraniol in strawberries and therefore matrix-matched standards were used in this matrix.

Conclusions

Geraniol is quantified in tomatoes, cucumber and strawberries using LC-MS/MS and a multi-residue method based on QuEChERS. Various extraction techniques were used.

Geraniol was successfully quantified in tomato, cucumber and strawberry and the methods proposed have been fully validated. Specificity of the method and absence of interference from the test item, internal standard or the various matrices was demonstrated, moreover two mass transition were followed and validated to confirm the identity of geraniol. The Limit of Quantification was 0.05 mg/kg (corresponding to 0.05 ppm) for geraniol in each matrix (tomatoes, cucumber and strawberries).

The methods presented herewith are satisfactory and can be applied to quantify geraniol residues in the described matrices (high water and high acid fruits).

Assessment and conclusion by applicant:

The validation of the methods for analysis for geraniol in tomato, cucumber and strawberry was not previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The methods are acceptable for the quantification of geraniol in tomato, cucumber (high water fruits) and strawberry (high acid fruits).

A 2.2.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

KCP 5.2/08 (A 2.2.2.2/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in body tissue (meat and liver) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination eugenol, methyl-eugenol, thymol and geraniol from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4.</p> <p>The LOQ is the lowest validated fortification level for eugenol, methyl-eugenol, thymol and geraniol and was thus successfully established at 0.01 mg/kg in meat and liver for all selected MS fragment ions or mass transitions.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20% and thereby comply with the standard acceptance criteria of the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.2/08 (A 2.2.2.2/01 of this dRR)
Report author	Driss F
Report year	2021f
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body tissue (meat and liver)
Report No	S20-06625
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4

SANCO/825/00 rev.8.1
ENV/JM/MONO(2007)17

Deviations from current test guideline None

Previous evaluation No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number SHBL2152
Substance content:	99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch:	19 August 2021
Storage conditions:	Ambient

Analysis parameters for meat

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz
Purge Flow	50 mL/min at 0.05 min
Injection volume	1 µL (depending on sensitivity)
Injector temperature	250°C
Column oven temperature programme	

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:	Approx. 13.4 min for Geraniol
Ionisation mode	Electron Impact Ionisation (EI)
Scan type	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Solvent delay	6 min
Ions monitored	Geraniol: 123 # (100 ms dwell), 69 (100 ms dwell), 41 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Analysis parameters for liver

Method type	LC-MS/MS
HPLC system	LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar) LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column	Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure	200 bar
Autosampler temperature	4°C
Column oven temperature	60°C
Injection volume	10 µL (depending on sensitivity)
Mobile phases	Eluent A: Acetonitrile; Eluent B: Water containing 5mM of ammonium acetate
Gradient	

Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
0.0	25	75	0.6
0.5	25	75	0.6
3.0	95	5.0	0.6
4.5	95	5.0	0.6
5.5	25	75	0.6
7.5	25	75	0.6

Divert valve	0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste
Retention time(s)	Approx. 2.90 min for Geraniol

Mass spectrometric conditions

MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer) SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	5500 V					
Ionspray turbo heater (TEM)	100°C					
Curtain gas (CUR)	Nitrogen set at [25-40] psi	Gas flow 1 (GS1)	Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)			
Collision gas (CAD)	Nitrogen set at 8 psi	Gas flow 2 (GS2)	Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)			
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Geraniol	172 → 81 #	7	10	15	10	100
	172 → 79	7	10	35	7	150

proposed (and/or used) for quantification but both of the mass transitions listed can be used for quantification

Sample preparation

Meat: An amount of 10 g ± 0.1 g of homogenised meat sample was weighed into a 50-mL centrifuge

tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube is then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. the contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Liver: An amount of 10 g ± 0.1 g of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 µL of toluene is added; the supernatant was evaporated to 600 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 µL.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.125 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.25 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 103 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25 %, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ion transitions by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Geraniol (%)		
Meat	80	<i>m/z</i> 123	<i>m/z</i> 69	<i>m/z</i> 41
		(-) 2.1	15.4	51.2
Liver	80	<i>m/z</i> 172→81	<i>m/z</i> 172→79	-
		(-) 16.8	(-) 20.6	-

Matrix effects were $\geq \pm 20\%$ and deemed to be significant for all analytes in meat and liver. Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL, corresponding to analyte concentrations of 0.025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver. Linearity was confirmed for each ion.

Ion	Meat
<i>m/z</i> 123 (GC-MS)	$y = 67x + 1496$ $r = 0.9996, r^2 = 0.9992$
<i>m/z</i> 69 (GC-MS)	$y = 926x + 24449$ $r = 0.9993, r^2 = 0.9986$
<i>m/z</i> 41 (GC-MS)	$y = 854x + 37878$ $r = 0.9995, r^2 = 0.9989$
Transition	Liver
<i>m/z</i> 172→81	$y = 285x + 1639$ $r = 0.9997, r^2 = 0.9993$
<i>m/z</i> 172→79	$y = 50x + 201$ $r = 0.9996, r^2 = 0.9993$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and precision was demonstrated. The LOQ is 0.01 mg/kg for meat and liver.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg in meat and 0.0027 mg/kg in liver.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 123							
Meat	0.01	81; 97; 89; 81;101	90	10	5	87	11
	0.1	91; 95; 67; 87; 84	85	13	5		
Fragment m/z 69							
Meat	0.01	87, 100; 89; 83; 105	93	10	5	89	11
	0.1	91; 97; 69; 88; 85	86	12	5		
Fragment m/z 41							
Meat	0.01	92; 102; 98; 78; 92	92	10	5	88	12
	0.1	90; 95; 65; 86; 84	84	14	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery	Overall Rel. Std. Dev.

						(%)	(%)
Transition m/z 172→ 81							
Liver	0.01	88, 89, 92, 88, 98	91	5	5	95	6
	0.1	98, 100, 105, 97, 95	99	4	5		
Transition m/z 172→ 79							
Liver	0.01	106, 90, 95, 91, 105	97	8	5	97	6
	0.1	98, 96, 104, 94, 91	97	5	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis of samples of matrix-matched standards of geraniol, control matrix, and control matrix spiked with geraniol. Three fragment ions were monitored by GC/MS for geraniol in meat and two ion transitions were monitored by LC-MS/MS in liver.

Additionally, a reagent blank and two control samples per matrix/analyte were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of geraniol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of geraniol in any investigated matrix meat and liver, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 103 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for geraniol re-analysed extracts were out of the range 70-110% and ± 20 % of the original result. Therefore, final extracts of meat and liver are considered to be unstable for 17 and 13 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Geraniol - Fragment <i>m/z</i> 123						
Meat	0	0.01	81;97;89;81;101	90	10	(+)22
	17		118;106; 99;116 [#]	110	8	
Transition <i>m/z</i> 172→ 81						
Liver	0	0.01	88;89;92;88;98	91	5	(-)29
	13		56;71;66;67 [#]	65	10	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

One value excluded according to Dixon's test

Extraction efficiency:

Not required as part of this study.

Conclusions

Geraniol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for meat samples and by LC-MS/MS following two ion transitions for liver samples. The method is specific.

This analytical method for the determination of geraniol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify geraniol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of geraniol in meat and liver.

A 2.2.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

KCP 5.2/09 (A 2.2.2.3/01 of this dRR)

The DT₉₀ of ~~eugenol and methyleugenol~~-geraniol in soil is less than three days and therefore this study is no longer required.

A 2.2.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

KCP 5.2/09 (A 2.2.2.4/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in surface water was not previously evaluated at EU level.</p> <p>The methods were successfully validated and is considered suitable for the determination of residues of thymol, methyl-eugenol, geraniol and eugenol in surface water with an LOQ of 0.1 µg/L. Any of the three ions validated for thymol, geraniol and methyl-eugenol are suitable for quantification and/or confirmation. For eugenol, either of the two HPLC columns are suitable for quantification and/or confirmation.</p> <p>The LOQ was 0.1 µg/L.</p> <p>No significant matrix effects were observed therefore calibration solutions for thymol, methyl-eugenol and geraniol were prepared in solvent.</p> <p>All mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for methyl-eugenol, thymol and geraniol are within 70 – 120% with relative standard deviations ≤ 20%.</p> <p>Satisfactory accuracy and precision results were achieved for eugenol on two HPLC columns of differing chemistry (C₁₈ and pentafluorophenyl), monitoring a single transition; mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for eugenol, are within 70 – 120% with RSD ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/09 (A 2.2.2.4/01 of this dRR)
Report author	Chambers J.
Report year	2020a
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Surface Water
Report No	GW/19/001
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1

	ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Geraniol is extracted from surface water via steam distillation and quantified by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to eugenol, geraniol, thymol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Name:	Geraniol analytical grade
AS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV6051
Active substance content:	99.4% w/w (equivalent to 994 g/kg)
Expiry date of lot/batch:	July 2020
Storage conditions:	Refrigerated

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 20°C/min to 250°C
Transfer line temperature:	280°C
Injection volume	2 µL
Injector inlet temperature:	250°C Splitless
Carrier gas:	Helium
Flow rate:	2.6 mL/min
Acquisition type:	SIM
Retention time:	Geraniol: approx. 5.95 min
Monitored ions	Geraniol: 69, 121 and 123 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

500 mL surface water were transferred to a 500 mL round bottom flask. After fortification, anti-bumping granules were added and 10 mL sample transferred to a liquid-liquid extractor followed by 5 mL of hexane/ethyl acetate (1:1 v/v). The flask was attached to the liquid-liquid extractor, placed on a heating mantle, connected to a condenser and switched on. After the sample reached boiling point and when the vapour was observed to be condensing above the liquid-liquid extractor, the procedure ran for 90 minutes. After cooling, the upper organic layer was transferred by pipette to a graduated tube and 1 mL trimethylpentane added.

The remaining contents of the liquid-liquid extractor were transferred to a second graduated tube and partitioned with 2 mL hexane/ethyl acetate (1:1 v/v) and the upper organic layer was combined with the first. The tube was placed a dri-block and evaporated until < 1 mL remained. The contents were quantitatively transferred to a 1.0 mL volumetric flask and made up to the mark with hexane/ethyl acetate (1:1 v/v). An aliquot was transferred to an auto-sampler vial for determination by GC-MS.

For recovery samples fortified at 0.1 µg/L and 1.0 µg/L, final extract concentrations are 50 ng/mL and 500 ng/mL, respectively.

Stock solutions and calibration standards (include actual concentrations)

Geraniol Standard Solution: Geraniol, 5 mg is accurately weighed and dissolved in acetone to produce a 1000 µg/mL stock solution. This stock solution is used to prepare 8 calibration standards spanning the concentration range 10 to 1500 ng/mL in trimethyl pentane.

No significant matrix effects were observed therefore calibration solutions for geraniol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 1 µg/mL and 10 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile. These fortification solutions are used to produce samples fortified at 0.1 µg/L and 1.0 µg/L. Fortified samples are then extracted and processed as described in sample preparation above. Final extract concentrations are 50 ng/mL and 500 ng/mL for 0.1 µg/L and 1.0 µg/L fortifications, respectively.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a $1/x$ weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f}{m \times V_s} \mu\text{g/L}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

V_s = sample volume

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/L}]}{\text{Amount spiked } [\mu\text{g/L}]} \times 100$$

Three ions were selected for geraniol.

Confirmation of substance identification

Confirmation method for the geraniol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 69, 121, 123) over the concentration range 10 ng/mL to 1000 ng/mL, corresponding to sample concentrations of 0.02 µg/L to 2.0 µg/L; seven standards were quantified:

69 m/z	121 m/z	123 m/z
Y = 98.93749x – 432.28202 R ² = 0.99888 r = 0.999173	Y = 6.07939x + 27.91896 R ² = 0.99951 r = 0.999317	Y = 12.46135x – 24.03819 R ² = 0.9979 r = 0.999865

The linearity of the method is considered validated for this range of concentration of geraniol.

Specificity:

Specificity of the analytical method was provided by the analysis of control matrix samples by GC-MS while monitoring three ions per analyte. The fragmentation observed in the geraniol mass spectrum

showed a base peak of m/z 69 but other fragments greater than m/z 100 were of low abundance. Consequently, m/z 69 was selected as the quantification ion for the geraniol method along with two ions > m/z 100 as confirmatory. No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of geraniol in 2 out of three determinations for the geraniol quantification ion m/z 69 but levels in the confirmatory ions were >30% LOQ which had an impact on the concentrations found in the fortification samples. However, subtraction of the levels found in controls from those in fortified samples gave satisfactory recovery efficiency results.

Copies of relevant chromatograms are provided for standards of geraniol, control surface water, and control surface water spiked with geraniol for each ion monitored. Moreover a mass spectrum of geraniol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.1 µg/L.

The limit of detection (LOD) was 0.02 µg/L (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in surface water and three ions were monitored.

	Geraniol m/z 121			Geraniol m/z 123			Geraniol m/z 69		
Spike (µg/L)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.1	97	13.7	5	94	16.0	5	104	8.8	5
1	83	9.8	9	88	5.2	9	84	5.0	9
Overall	88	13.8	14	90	10.7	14	91	12.7	14

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability of the analyte in solvent and matrix was determined. Geraniol was found stable in solvent for 55 days, and geraniol extracts were found to be stable for 22 days when stored at 4-6°C.

Conclusions

The geraniol is extracted in surface water via steam distillation and quantified by GC-MS (three ions monitored).

Geraniol was successfully quantified in surface water and the method proposed has been fully validated. Specificity of the method and absence of interference from the test item, internal standard or surface water was demonstrated. The Limit of Quantification was 0.1 µg/L for geraniol in surface water.

The method presented herewith is satisfactory and can be applied to quantify geraniol in surface water.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in surface water was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of geraniol in surface water.

A 2.2.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

KCP 5.2/10 (A 2.2.2.5/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in air sampling cartridges was not previously evaluated at EU level.</p> <p>The method was successfully validated and is considered suitable for the determination of residues of thymol, eugenol, methyl-eugenol and geraniol in air with an LOQ of 1.2 µg/m³</p>
-------------------	---

	<p>according to guidance documents SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours. All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%. The study is acceptable.</p>
--	--

Data point:	CP 5.2/10 (A 2.2.2.5/01 of this dRR)
Report author	Chambers J.
Report year	2020b
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Air
Report No	TS/19/003
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Residues of geraniol were extracted from pre-packed XAD-2 cartridges by sonication with ethyl acetate and final determination was performed by GC-MS, monitoring three ions of m/z >100.

Although the study report refers to eugenol, geraniol, thymol and methyleugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Name:	Geraniol analytical standard
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number BCBV6051
Active substance content:	99.4% w/w (equivalent to 9940 g/kg)
Expiry date of lot/batch:	July 2020
Storage conditions:	Refrigerated

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	Agilent CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 12.5°C/min to 215°C and hold for 1 minute
Transfer line temperature:	280°C
Injection volume	3 µL
Injector inlet temperature:	280°C pulsed-splitless
Carrier gas:	Helium
Flow rate:	2.0 mL/min
Acquisition type:	SIM
Retention time:	Geraniol: approx. 4 min

Monitored ions Geraniol: 121, 123, 139* and 111* m/z

* m/z 139 used for extraction efficiency test and m/z 111 used for all other tests

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

If the entire contents of the cartridge are required for analysis, transfer the front and rear segments of the cartridge to a screw-top vial including any glass-wool dividers.

If the front and rear segments of the cartridge are to be analysed separately, transfer the front segment of the cartridge to a screw-top vial including any glass-wool dividers before and after the sorbent, and transfer the rear segments of the cartridge to a screw-top vial including any glass-wool dividers after the sorbent, taking care not to pass the rear section through the front section to avoid contamination.

Add ethyl acetate (5 x 1 mL) extraction solvent to the empty cartridge, collecting all washes into the (front segment) vial. Vortex the sample for 1 minute, sonicate the sample for 10 minutes and vortex the sample for 30 seconds. Transfer an aliquot into an auto-sampler vial and quantify by GC-MS.

For recovery samples fortified at 0.5 µg/cartridge and 5.0 µg/cartridge, final extract concentrations are 100 ng/mL and 1000 ng/mL, respectively.

Stock solutions and calibration standards

Geraniol stock solution: geraniol, 5 mg, is accurately weighed and dissolved in acetonitrile to produce a 1000 µg/mL stock solution. This stock solution is further diluted in ethyl acetate to produce an intermediate stock from which calibration standards in ethyl acetate are produced. The calibration solutions span the concentration range 20 to 1300 ng/mL in ethyl acetate (corresponding to 0.24 to 15.6 µg/m³).

No significant matrix effects were observed therefore calibration solutions for geraniol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 200 µg/mL and 20 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile.

The target LOQ for each analyte in air was 1.2 µg/m³. The limit of quantification (LOQ) was taken as the lowest fortification level where an acceptable mean recovery is obtained in the range 70 to 110% with an RSD < 20%.

The rate of air flow was 1.0 L/min for a total duration of 7 hours. This resulted in a volume of 420 L or 0.42 m³ over the 420-minute period, equating to a fortification level of 0.504 µg/cartridge, rounded to 0.50 µg/cartridge (0.42 m³ x 1.2 µg/m³).

The two ends of each cartridge were broken off using a tube cutter. The front segment of each cartridge was fortified at 0.5 µg/cartridge (LOQ) by pipetting 25 µl of the 20 µg/mL fortification solution and at 5 µg/cartridge (10 x LOQ) by pipetting 25 µl of the 200 µg/mL fortification solution.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a 1/x weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f \times D}{m \times 1000} \mu\text{g/cartridge}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

D = Dilution factor

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/cartridge}]}{\text{Amount spiked } [\mu\text{g/cartridge}]} \times 100$$

Four ions were selected for geraniol (m/z 121, 123, 139 and 111 with m/z 139 used for extraction efficiency test and m/z 111 used for all other tests).

Confirmation of substance identification

Confirmation method for geraniol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 121, 123, 139 and 111 with m/z 139 used for extraction efficiency test and m/z 111 used for all other tests) over the concentration range 20 ng/mL to 1300 ng/mL, corresponding to sample concentrations of 0.24 µg/m³ to 15.6 µg/m³; eight standards were quantified:

111 m/z	121 m/z	123 m/z	139 m/z
Y = 11.72764x - 6.21668 R ² = 0.99989 r = 0.999858	Y = 9.9999x + 11.31762 R ² = 0.99983 r = 0.999921	Y = 20.7041x - 17.4019 R ² = 0.99982 r = 0.999894	Y = 10.66373 - 104.31288 R ² = 0.99863 r = 0.998682

The linearity of the method is considered validated for this range of concentration of geraniol.

Specificity:

Specificity of the analytical method was provided by the analysis of control matrix samples by GC-MS while monitoring four ions above m/z 100. No interference or contamination peak was detected above 30% LOQ at the same retention time of geraniol in any control or blank samples from the extraction efficiency tests.

During the retention capacity tests, levels greater than 30% LOQ were observed in some geraniol control samples. Correspondingly higher levels of Geraniol were observed in the front segment (>110% of the fortification level) but satisfactory recoveries were obtained after subtraction of the control sample.

Copies of relevant chromatograms are provided for standards of geraniol, control cartridge (front segment), and cartridge (front and back segment) spiked with geraniol for each ion monitored. Moreover a mass spectrum of geraniol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours.

The limit of detection (LOD) was 0.15 µg/cartridge based on 30% LOQ (lowest calibration level) and equates to 0.36 µg/m³.

Accuracy, Retention capacity, Repeatability (precision):

Accuracy and repeatability (procedural recoveries) were assessed at two levels of concentrations in cartridge and three ions were monitored.

	Geraniol m/z 121			Geraniol m/z 123			Geraniol m/z 111		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.5	99	-	2	102	-	2	101	-	2
5.0	101	-	2	101	-	2	101	-	2
Overall	100	1.4	4	102	0.3	4	101	0.3	4

Retention capacity was assessed at two levels of concentrations in cartridge and three ions were monitored.

	Geraniol m/z 121			Geraniol m/z 123			Geraniol m/z 111		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.5	91	5.4	6	92	6.7	6	85	6.9	6
5	96	1.8	6	97	1.7	6	97	1.7	6
Overall	93	4.8	12	94	5.2	12	91	8.3	12

Tests with geraniol ion m/z 139 resulted in interference in control and treated samples therefore ion m/z 111 was substituted for m/z 139 in subsequent tests.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Extraction efficiency:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with geraniol at 5 µg/cartridge (10xLOQ) which achieved mean recovery efficiencies from 6 replicate cartridges of 95%, 94% and 95% for geraniol ions m/z 121, 123 and 139, respectively.

Cartridge stability:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with each analyte at 5 µg/cartridge (10xLOQ) and stored in the dark under ambient conditions for 8 days, which achieved mean recovery efficiencies from three replicate cartridges of 96%, 96% and 96% for geraniol ions m/z 121, 123 and 139, respectively.

Stability:

Solvent standards were shown to be stable in solvent solutions stored for 10 and 65 days at nominally 4°C and extract solutions showed no degradation when stored for at least 18 days.

Conclusions

Geraniol was successfully quantified in air sampling cartridges (pre-packed XAD-2 cartridges) following extraction with ethyl acetate and quantification by GC-MS (three ions monitored) and the method proposed has been fully validated for linearity, accuracy and precision. Specificity of the method and absence of interference from the test item, or the matrix was demonstrated. The Limit of Quantification was 1.2 µg/m³ for geraniol content in air.

The method presented herewith is satisfactory and can be applied to quantify geraniol in air via air sampling cartridges.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in air sampling cartridges was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of geraniol in air via air sampling cartridges.

A 2.2.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

KCP 5.2/07 (A 2.2.2.6/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in body fluids (plasma and urine) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination of eugenol, geraniol, thymol and methyl-eugenol on plasma and urine from the tested LOQ of 0.01 mg/L up to 0.1 mg/L according to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev.4.</p> <p>The LOQ is 0.01 mg/kg for eugenol, geraniol, thymol and methyl-eugenol for plasma and urine.</p> <p>All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point: CP 5.2/07 (A 2.2.2.6/01 of this dRR)

Report author Driss F.

Report year	2021e
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body fluid (plasma and urine)
Report No	S20-06626
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

In brief, samples of body fluids (plasma and urine) were extracted with acetonitrile. A salt mixture containing magnesium sulphate sodium chloride (urine) or magnesium sulphate, sodium chloride and sodium citrate (plasma) was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name:	Geraniol analytical grade
CAS number:	106-24-1
Source and lot/batch no.:	Sigma Aldrich, batch number SHBL2152
Substance content:	99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch:	19 August 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-1701 (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz
Purge Flow	50 mL/min at 0.05 min
Injection volume	1 µL (depending on sensitivity)
Injector temperature	250°C
Column oven temperature programme	

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:	Approx. 13.4 min for geraniol
Ionisation mode	Electron Impact Ionisation (EI)
Scan type	SIM (Selected Ion Monitoring)

Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Geraniol: 123# (100 ms dwell), 69 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Chromatographic conditions for confirmation of geraniol

Method type LC-MS/MS
HPLC system LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar)
LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure 200 bar
Autosampler temperature 4°C
Column oven temperature 60°C
Injection volume 10 µL (depending on sensitivity)
Mobile phases Eluent A: Acetonitrile;
Eluent B: Water containing 5mM of ammonium acetate
Gradient

Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
0.0	25	75	0.6
0.5	25	75	0.6
3.0	95	5.0	0.6
4.5	95	5.0	0.6
5.5	25	75	0.6
7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste

Retention time(s) Approx. 2.90 min for Geraniol

Mass spectrometric conditions

MS system SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)
SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation type Electrospray ionisation (ESI, TurboIonSpray)
Polarity Positive ion mode
Scan type MS/MS, Multiple Reaction Monitoring (MRM)
Capillary voltage (IS) 5500 V
Ionspray turbo heater 100°C
(TEM)
Curtain gas (CUR) Nitrogen set at [25-40] Gas flow 1 (GS1) Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)
Collision gas (CAD) Nitrogen set at 8 psi Gas flow 2 (GS2) Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)
Analyte monitored Mass transition monitored (m/z) Declustering potential (DP) [V] Entrance potential (EP) [V] Collision energy (CE) [eV] Cell exit potential (CXP) [V] Dwell time [ms]

Geraniol	172 → 81 (pos)	7	10	15	10	100
----------	-------------------	---	----	----	----	-----

Sample preparation

Urine: An amount of 10 g ± 0.1 g of homogenised urine sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 10 mL acetonitrile was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

The supernatant was transferred into a polypropylene tube and 2 tubes containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL was transferred a centrifuge tube and 100 µL of toluene is added. The supernatant was evaporated down to 300 µL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 800 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 0.75 µg/mL.

Plasma: An amount of 5 g ± 0.1 g of homogenised plasma sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. The centrifuge tube was capped and vortexed for 5 min. For extraction, exactly 5.0 mL of acetonitrile were added. The centrifuge tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 1 minute. ½ citrate extraction tube containing 4 g of magnesium sulfate, 1 g of sodium chloride was added and the tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 5 minutes. The sample tube was centrifuged for 10 minutes at 4000 rpm at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 3.0 mL is transferred a centrifuge tube and 50 µL of toluene is added; the supernatant was evaporated to 300 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 400 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 0.75 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of matrix which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0027 mg/kg to 0.532 mg/kg in both matrices).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix plasma and urine on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard

A _{Matrix-Std}	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
C _{Solv-Std}	Nominal concentration of solvent standard in ng/mL
C _{Matrix-Std}	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 103 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C _A	Concentration of analyte in final extract (ng/mL)
A _A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000133 for plasma and urine	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ions by GC/MS plus one ion transition by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Geraniol (%)		
		m/z 123	m/z 69	m/z 172→81
Plasma	80	(+)61.1	(+)38.1	(+)0.7
Urine	80	(+)36.4	(+)21.9	(-)0.3

For plasma and urine, matrix effects on the detection of Geraniol in extracts were found to be significant ($\geq 20\%$). Therefore matrix-matched standards were used for quantification.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL, corresponding to analyte concentrations of 0.0027 mg/kg to 0.532 mg/kg in plasma and urine. Linearity was confirmed for each ion.

	Plasma	Urine
m/z 123 (GC-MS)	y = 72 x + 1825 r = 0.9987, r ² = 0.9974	y = 55 x + 736 r = 0.9985, r ² = 0.9971
m/z 69 (GC-MS)	y = 677 x + 20619 r = 0.9988, r ² = 0.9976	y = 527 x + 7273 r = 0.9985, r ² = 0.9971
m/z 172→81 (LC-MS/MS)	y = 0.563 x - 1746 r = 0.9992, r ² = 0.9985	y = 417 x + 2492 r = 0.9981, r ² = 0.9962

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg for Plasma and urine.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0027 mg/kg in plasma and urine.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in plasma and urine matrix.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 123							
Plasma	0.01	93, 97, 114, 75, 112	98	16	5	98	14
	0.1	83, 91, 106, 115, 94	98	13	5		
Urine	0.01	114, 118, 113, 92, 113	110	9	5	101	12
	0.1	98, 86, 86, 100, 90	92	7	5		
Fragment <i>m/z</i> 69							
Plasma	0.01	96, 107, 113, 65, 11	98	20	5	101	17
	0.1	83, 92, 118, 120, 105	104	15	5		
Urine	0.01	108, 109, 115, 82, 119	107	14	5	99	8
	0.1	100, 88, 85, 101, 87	92	8	5		
Mass Transition <i>m/z</i> 172→81							
Plasma	0.01	80, 82, 83, 77, 84	81	3	5	82	4
	0.1	80, 81, 80, 86, 88	83	5	5		
Urine	0.01	108, 111, 106, 101, 117	109	92	5	100	10
	0.1	98, 87, 87, 95, 91	5	5	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of geraniol, control matrix, and control matrix spiked with geraniol. Two fragment ions and one selected mass transition were monitored.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of geraniol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix plasma and urine, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 103 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Geraniol was found to be stable in final extracts of plasma for 8 days, when stored at typically 1°C to 10°C in the dark. However, urine re-analysed extracts were out of the range of $\pm 20\%$ of the original results. Therefore, samples should be analysed as quickly as possible after extraction.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Geraniol - Fragment <i>m/z</i> 123						
Plasma	0	0.01	93, 97, 114, 75, 112	98	16	7
	8		93, 94, 118, 87, 135	105	19	
Geraniol – Mass transition 172→81						
Urine	0	0.01	105, 113, 107, 112, 106	109	3	27
	10		137, 140, 131, 141, 139	138	3	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Geraniol is extracted from plasma or urine matrix using acetonitrile, then quantified by GC-MS using two separate ions and by LC-MS/MS using one ion transition for quantification and confirmation of method specificity.

This analytical method for the determination of geraniol content in body fluid matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in both plasma and urine.

The method presented herewith is satisfactory and can be applied to quantify geraniol in plasma and urine.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in body fluids (plasma and urine) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated. The method is acceptable for the quantification of geraniol in plasma and urine.

KCP 5.2/08 (A 2.2.2.6/02 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for geraniol in body tissue (meat and liver) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination eugenol, methyl-eugenol, thymol and geraniol from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4.</p> <p>The LOQ is the lowest validated fortification level for eugenol, methyl-eugenol, thymol and geraniol and was thus successfully established at 0.01 mg/kg in meat and liver for all selected MS fragment ions or mass transitions.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations $\leq 20\%$ and thereby comply with the standard acceptance criteria of the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/08 (A 2.2.2.6/02 of this dRR)
Report author	Driss F
Report year	2021f
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyleugenol in body tissue (meat and liver)
Report No	S20-06625
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only geraniol is considered in this summary.

Materials and methods

Analytical grade geraniol was used as test item.

Test material

Test Standards

Name: Geraniol analytical grade
CAS number: 106-24-1
Source and lot/batch no.: Sigma Aldrich, batch number SHBL2152
Substance content: 99.1% w/w (equivalent to 991 g/kg)
Expiry date of lot/batch: 19 August 2021
Storage conditions: Ambient

Analysis parameters for meat

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C
Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 13.4 min for Geraniol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Geraniol: 123 # (100 ms dwell), 69 (100 ms dwell), 41 (100 ms dwell)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Analysis parameters for liver

Method type LC-MS/MS
HPLC system LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar)
LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure 200 bar
Autosampler temperature 4°C
Column oven temperature 60°C
Injection volume 10 µL (depending on sensitivity)
Mobile phases Eluent A: Acetonitrile;
Eluent B: Water containing 5mM of ammonium acetate
Gradient

Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
0.0	25	75	0.6
0.5	25	75	0.6
3.0	95	5.0	0.6
4.5	95	5.0	0.6
5.5	25	75	0.6
7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste

Retention time(s) Approx. 2.90 min for Geraniol

Mass spectrometric conditions

MS system SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)
SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type Electrospray ionisation (ESI, TurboIonSpray)

Polarity Positive ion mode

Scan type MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage (IS) 5500 V

Ionspray turbo heater 100°C
(TEM)

Curtain gas (CUR) Nitrogen set at [25-40] psi Gas flow 1 (GS1) Nitrogen set at 60 psi
(+10 psi if TripleQuad 6500 system used)

Collision gas (CAD) Nitrogen set at 8 psi Gas flow 2 (GS2) Nitrogen set at 40 psi
(+10 psi if TripleQuad 6500 system used)

Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Geraniol	172 → 81 #	7	10	15	10	100
	172 → 79	7	10	35	7	150

proposed (and/or used) for quantification but both of the mass transitions listed can be used for quantification

Sample preparation

Meat: An amount of 10 g ± 0.1 g of homogenised meat sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube is then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. the contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection. For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.0 µg/mL.

Liver: An amount of 10 g ± 0.1 g of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube

is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 µL of toluene is added; the supernatant was evaporated to 600 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 µL.

For samples fortified at 0.01 mg/kg, geraniol concentration in the final extract was 0.125 µg/mL, and for samples fortified at 0.1 mg/kg, geraniol concentration in the final extract was 1.25 µg/mL.

Stock solutions and calibration standards

Geraniol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \cdot C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Stock solutions stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 103 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25 %, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C _A	Concentration of analyte in final extract (ng/mL)
A _A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ion transitions by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Geraniol (%)		
		m/z 123	m/z 69	m/z 41
Meat	80	(-) 2.1	15.4	51.2
Liver	80	m/z 172→81	m/z 172→79	-
		(-) 16.8	(-) 20.6	-

Matrix effects were $\geq \pm 20\%$ and deemed to be significant for all analytes in meat and liver. Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL, corresponding to analyte concentrations of 0.025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver. Linearity was confirmed for each ion.

Ion	Meat
-----	------

<i>m/z</i> 123 (GC-MS)	$y = 67x + 1496$ $r = 0.9996, r^2 = 0.9992$
<i>m/z</i> 69 (GC-MS)	$y = 926x + 24449$ $r = 0.9993, r^2 = 0.9986$
<i>m/z</i> 41 (GC-MS)	$y = 854x + 37878$ $r = 0.9995, r^2 = 0.9989$

Transition	Liver
<i>m/z</i> 172→81	$y = 285x + 1639$ $r = 0.9997, r^2 = 0.9993$
<i>m/z</i> 172→79	$y = 50x + 201$ $r = 0.9996, r^2 = 0.9993$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and precision was demonstrated. The LOQ is 0.01 mg/kg for meat and liver.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg in meat and 0.0027 mg/kg in liver.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 123							
Meat	0.01	81; 97; 89; 81; 101	90	10	5	87	11
	0.1	91; 95; 67; 87; 84	85	13	5		
Fragment <i>m/z</i> 69							
Meat	0.01	87, 100; 89; 83; 105	93	10	5	89	11
	0.1	91; 97; 69; 88; 85	86	12	5		
Fragment <i>m/z</i> 41							
Meat	0.01	92; 102; 98; 78; 92	92	10	5	88	12
	0.1	90; 95; 65; 86; 84	84	14	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

Geraniol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 172→ 81							
Liver	0.01	88, 89, 92, 88, 98	91	5	5	95	6
	0.1	98, 100, 105, 97, 95	99	4	5		
Transition <i>m/z</i> 172→ 79							
Liver	0.01	106, 90, 95, 91, 105	97	8	5	97	6
	0.1	98, 96, 104, 94, 91	97	5	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis of samples of matrix-matched standards of geraniol, control matrix, and control matrix spiked with geraniol. Three fragment ions were monitored by GC/MS for geraniol in meat and two ion transitions were monitored by LC-MS/MS in liver.

Additionally, a reagent blank and two control samples per matrix/analyte were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of geraniol. For all fragment ions or mass transition, the samples showed no significant

interference above 30 % of LOQ at the retention time of geraniol in any investigated matrix meat and liver, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 103 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for geraniol re-analysed extracts were out of the range 70-110% and ± 20 % of the original result. Therefore, final extracts of meat and liver are considered to be unstable for 17 and 13 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Geraniol - Fragment <i>m/z</i> 123						
Meat	0	0.01	81;97;89;81;101	90	10	(+)22
	17		118;106; 99;116 [#]	110	8	
Transition <i>m/z</i> 172→ 81						
Liver	0	0.01	88;89;92;88;98	91	5	(-)29
	13		56;71;66;67 [#]	65	10	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

One value excluded according to Dixon's test

Extraction efficiency:

Not required as part of this study.

Conclusions

Geraniol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for meat samples and by LC-MS/MS following two ion transitions for liver samples. The method is specific.

This analytical method for the determination of geraniol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for geraniol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify geraniol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for geraniol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of geraniol in meat and liver.

A 2.2.2.7 Other Studies/ Information

No new or additional studies have been submitted.

A 2.3 Analytical methods for thymol

A 2.3.1 Methods used for the generation of pre-authorization data (KCP 5.1) GRAPES (Residues)

KCP 5.1.2/01 (A 2.3.1/01 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/01 (A 2.3.1/01 of this dRR)
Report author	Bailey A.
Report year	2007
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern Europe (2006 – 2007)
Report No	AF/10728/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev 4
Deviations from current test guideline	The calibration range does not extend to 30% of the LOQ; Although three ions were monitored, except for specificity, validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with acetone. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and thymol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Fluka, lot n°1130656
Active substance content:	> 99.9%
Expiry date of lot/batch:	August 2007
Storage conditions:	-18°C.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25m x 0.32mm internal diameter fused silica capillary coated with PAS-1701

Oven:	(0.25µm film) 50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode,
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Thymol: Approx. 6.0 min
Monitored ions/transitions:	135 m/z (quantification), 150 and 115 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and acetone (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.01 and 0.1 mg/kg, extract concentrations are 0.005 µg/mL and 0.05 µg/mL respectively. For samples fortified at 0.05 and 0.2 mg/kg, extract concentrations are 0.025 µg/mL and 0.1 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for thymol. Eight calibration standards were quantified. The equation to the calibration line was $28495218x - 314942$ and the

correlation coefficient $R = 0.9994$. The method is linear.

Specificity:

Specificity was studied by analysis samples of standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol.

No residues at the retention time of thymol were found in untreated specimens taken from the control plots of each trial and subsequently used for procedural recovery tests.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol for each ion monitored. Full mass spectrometry scan of thymol under the described analytical conditions was performed to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.05 mg/kg of thymol residues in sample. The LOD was not stated.

Accuracy, Repeatability (precision):

Originally, it was expected that a limit of quantification of 0.01 mg/kg could be achieved therefore procedural recoveries were performed at levels of 0.01 and 0.1 mg/kg with each analytical batch. However due to problems associated with background interference and the inability to use three ions for quantification and qualification, the original target LOQ of 0.01 mg/kg could not be met. Following analysis of residue specimens, additional method validation, according to SANCO/3029/99 rev 4 (11/07/00), was performed by fortification of untreated grape specimens at 0.05 and 0.2 mg/kg with five replicates being analysed at each level. A composite untreated specimen from this study was prepared for this purpose. The analytical method was successfully validated at 0.05, 0.1 and 0.2 mg/kg.

Fortification level (mg/kg)	Mean recovery (%)	% RSD	n
Validation			
0.05	93	8.5	5
0.2	84	12.4	5
Combined	88	11.3	10
Procedural recoveries			
0.1	85	5.8	4

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability in the matrix was demonstrated by acceptable recoveries at 0.1 mg/kg fortification level.

Conclusions

Thymol residues are extracted from raw commodity grapes sample with acetone, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

This analytical method for the determination of thymol residues content in raw commodity grape samples has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. Although attempts to validate the method down to 0.01 mg/kg failed, the method was successfully validated at levels of 0.05 and 0.2 mg/kg. A full mass spectrometry scan of thymol under the described analytical conditions is presented as part of the study to confirm analyte identification. Three ions were monitored to confirm analyte identity. The method is acceptable for the quantification of thymol residues in raw commodity grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.

The method is acceptable for the quantification of thymol residues in grapes.

KCP 5.1.2/02 (A 2.3.1/02 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/02 (A 2.3.1/02 of this dRR)
Report author	Bailey A.
Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues on the surface of grapes by deposit analysis resulting from sequential applications of 3AEY, in Southern Europe (2006)
Report No	AF/11125/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC)
Deviations from current test guideline	Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 4.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Fluka, lot n°1130656 43905125

Active substance content:	> 99.9%
Expiry date of lot/batch:	August 2007
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25 m x 0.32 mm internal diameter fused silica capillary coated with PAS-1701 (0.25 µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode,
Carrier gas:	Helium

Flow rate: 1 mL/min
Ionization mode: Selected ion monitoring (SIM)
Retention time: Approx. 6.0 min
Monitored ions: 135 m/z (quantification), 150 and 115 m/z (qualification)

Sample preparation

Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.01, 0.1 and 1.0 mg/kg, extract concentrations are 0.01, 0.1 and 1.0 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen. Procedural recoveries were performed at 0.01 and 0.26 mg/kg, equivalent to 0.01 and 0.26 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for thymol. Eight calibration standards were quantified. The equation to the calibration line was $8082255x - 27106$ and the correlation coefficient $R = 0.9997$

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was studied by analysis samples of standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol.

No residues at the retention time of thymol were found in untreated specimens taken from the control plots of each trial and subsequently used for procedural recovery tests.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol for each ion monitored. Full mass

spectrometry scan of thymol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated. LOQ is 0.01 mg/kg of thymol residue in sample. The LOD was not stated.

Accuracy, Repeatability (precision):

Fortification level (mg/kg)	Mean recovery (%)	% RSD	n
Validation			
0.01	88	5.1	5
0.5	91	7.5	5
Combined	89	6.5	10
Procedural recoveries			
0.01	95	-	-
0.26	81	-	-
Mean	88	-	-

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability in the matrix was not investigated in this study.

Conclusions

Thymol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS (three ions monitored).

The method described was acceptably validated according to SANCO/3029/99 rev.4. Full mass spectrometry scan of thymol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification. Three ions were monitored to confirm analyte identity. The method is acceptable for the quantification of thymol in grapes. The Limit of Quantification was 0.01 mg/kg for thymol residues in grape samples.

The method presented herewith is satisfactory and can be applied to quantify thymol residues in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level. It was performed under GLP, followed Guideline SANCO/3029/99 rev.4 (2000) requirements and was successfully validated.

The method is acceptable for the quantification of thymol residues in grapes.

KCP 5.1.2/03 (A 2.3.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of geraniol, eugenol and thymol residues in grapes was not previously evaluated at EU level.</p> <p>The method has been validated for the determination of geraniol, eugenol and thymol residues in grapes according to SANCO/3029/99 rev.4.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with ≤ 20% RSD).</p> <p>The limit of quantification (LOQ) of the analytical method was 0.05 mg/kg of geraniol, eugenol and thymol residues in sample.</p> <p>The study has been accepted for the quantification of geraniol, eugenol and residue in grapes.</p>
-------------------	---

Data point:

CP 5.1.2/03 (A 2.3.1/03 of this dRR)

Report author

Cheshire A.

Report year	2008
Report title	To determine the magnitude of geraniol, eugenol and thymol residues at harvest in the raw agricultural commodity grapes resulting from sequential applications of 3AEY, in Southern and Northern France, 2007
Report No	AF/12268/ED
Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) ENV/JM/MONO(2007)17
Deviations from current test guideline	Although three ions were monitored, validation data is available on one ion only.
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and thymol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Fluka, lot n°1305941 32807516
Active substance content:	> 99.9%
Expiry date of lot/batch:	August 2009
Storage conditions:	4°C

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	30 m x 0.25 mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25 µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature:	Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature:	275°C
Injection volume:	Not stated
Injection mode:	Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Thymol: Approx. 18 min
Monitored ions/transitions:	135 m/z (quantification), 150 and 115 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed.

For samples fortified at 0.05 mg/kg, 0.1 mg/kg, 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg, extract concentrations are 0.025 µg/mL, 0.05 µg/mL, 0.125 µg/mL, 0.25 µg/mL and 0.5 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in ethyl acetate to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

Linearity:

Linearity was investigated over the range 0.0125 – 1.0 µg/mL for thymol. Seven calibration standards were quantified. The equation to the calibration line was $13438320x - 70117$ and the correlation coefficient $R = 0.9995$

Specificity:

Specificity was studied by analysis samples of standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest achieved acceptable recovery. LOQ is 0.05 mg/kg of thymol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL.

The LOD was not stated.

Accuracy, Repeatability (precision):

Accuracy was verified at five levels: 0.05, 0.1, 0.25, 0.5 and 1.0 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Thymol recovery (%)
Procedural recovery	
0.05	108
0.25	100
0.05	104
0.5	94
0.05	106
0.1	106
0.05	104
0.25	97
0.05	101
1.0	94
Mean	101
%RSD	5.0 (n = 10)

In addition, accuracy and precision were also verified at two levels through five independent sample fortifications at 0.05 and 1.0 mg/kg.

Fortification level (mg/kg)	Thymol recovery (%)	%RSD (n)
validation		
0.05	97	2.2 (n = 5)
1.0	100	0.5 (n = 5)
Overall	98	2.0 (n = 10)

Stability:

Stability of thymol residue in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Thymol residues are extracted from raw commodity grapes sample with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

This analytical method for the determination of thymol residues content in raw commodity grape samples has been acceptably validated by definition of the specificity, the linearity, the accuracy and the precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled.

The method is confirmed as acceptable for the quantification of thymol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residue in grapes was not previously evaluated at EU level. It was performed under GLP and complies with SANCO/3029/99 rev.4 guidance.

The method is acceptable for the quantification of thymol residue in grapes.

KCP 5.1.2/04 (A 2.3.1/04 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/04 (A 2.3.1/04 of this dRR)
Report author	Jones S.
Report year	2012
Report title	Determination of natural background level residues of thymol, eugenol and geraniol in grapes

Report No	S11-03787
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 EU 1999: 1607/VI/97 7032/VI/95 rev.5
Deviations from current test guideline	The method presented is in agreement with Guidance Documents SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4
Previous evaluation	Yes, evaluated and accepted in DAR (Addendum 2012) under data point IIA 6.3; IIIA 8.2
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with ethyl acetate. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and thymol residues are quantified by GC-MS.

The method described herewith follows the validated EAS (formally Agrisearch) method 'Thymol, Eugenol & Geraniol/Crops/DB/08/1'. The method was fully validated in EAS study AF/10728/ED and EAS study AF/12268/ED, which are included in this submission.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test Standards

Name:	Thymol crystals, Pure
Source and lot/batch no.:	Penta Manufacturing Company, lot n°115628
Active substance content:	99.3%
Expiry date of lot/batch:	15 October 2014
Storage conditions:	Refrigerated.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	30m x 0.25mm internal diameter fused silica capillary coated with SOL-GEL WAX (0.25µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 2°C/min – 210°C (1 min) – 40°C/min – 280°C (10 min)
Detector temperature:	Transfer line, 280°C; Source, 230°C; MS Quad, 150°C
Injector temperature:	275°C
Injection volume:	Not stated
Injection mode:	Splitless mode, temperature 275°C single taper liner, glass wool, deactivated, low pressure drop P/N 5183-4647
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Thymol: Approx. 16.7 min
Monitored ions/transitions:	135 m/z (quantification), 150 and 115 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and ethyl acetate (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. A 25 mL aliquot is transferred to a vial containing 5.0 g anhydrous sodium sulphate. The contents are shaken and allowed to settle. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS. The sample is stored frozen if not immediately analysed. For samples fortified at 0.05, 0.1 and 0.2 mg/kg, extract concentrations are 0.025, 0.05 and 0.1 µg/mL respectively.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.0125 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

Linearity was investigated over the range 0.0125 – 1.0 µg/mL. Seven calibration standards were quantified. The equation to the calibration line was $11289051x - 36679$ and the correlation coefficient $R = 1.0000$. The linearity of the method is considered validated for this range of concentration

Specificity:

Specificity was studied by analysis samples of standards of thymol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with thymol.

Interferences were not observed.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape (white and red) samples, untreated grape (white and red) samples spiked with thymol for each ion monitored.

LOD, LOQ:

The LOQ was defined as the lowest achieved acceptable recovery. LOQ is 0.05 mg/kg of thymol residues in sample, equivalent to a theoretical extract concentration of 0.025 µg/mL. Although the LOD was not stated, the lowest calibration standard concentration (0.0125 µg/mL, equivalent to a theoretical fortification level of 0.025 mg/kg is proposed as LOD.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 0.05 mg/kg, 0.1 mg/kg and 0.2 mg/kg through procedural recoveries.

Fortification level (mg/kg)	Thymol recovery (%)
0.05	95
0.2	107
0.05	105
0.1	109
Mean	104
%RSD	6.0

Stability:

Stability of thymol residues in the matrix was not investigated as samples were analysed within 2 days of extraction.

Conclusions

Thymol residues are extracted from raw commodity grape samples with ethyl acetate, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission. The Limit of Quantification was 0.05 mg/kg for thymol residues in grape samples. This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of thymol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007) and AF/12268/ED (Cheshire, 2008), which are included in this submission.

The method is acceptable for the quantification of thymol residues in grapes.

KCP 5.2/03 (A 2.3.1/05 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.2/03 (A 2.3.1/05 of this dRR)
Report author	Brown D.
Report year	2007
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, and 6 months (2006-2007)
Report No	AD/11145/ED

Document No	Not applicable
Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) SANCO/3029/99 rev.4 SANCO/825/00 rev.7 EU Working Document 7032/VI/95 rev.5
Deviations from current test guideline	The calibration range does not extend to 50% of the LOQ; Although three ions were monitored, except for specificity validation data is available on one ion only.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 6.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

The method was validated prior to use and validation data is presented in Agrisearch study AF/11125/ED, which is included in this submission and was performed in the same test facility.

Materials and methods

Test material

Test Standards

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Fluka, lot n°1130656
Active substance content:	99.9%
Expiry date of lot/batch:	31 August 2007
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Hewlett-Packard 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	25 m x 0.32 mm internal diameter fused silica capillary coated with PAS-1701 (0.25 µm film)
Oven:	50°C (2 min) – 20°C/min – 280°C (15 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume:	Not stated
Injection mode:	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode:	Selected ion monitoring (SIM)
Retention time:	Thymol: Approx. 13 min
Monitored ions:	164 m/z (quantification), 149 and 131 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks. The analysis is designed to quantify surface residue only, therefore samples are de-stalked but not homogenised.

Extraction: A 500 g subsample is transferred to a 2000 mL beaker and acetone (500 mL) is added. Fortification is performed at this point if necessary. The sample is sonicated for 10 minutes to remove any

surface deposit and the extract is decanted. The process is repeated, the extracts are combined and adjusted to 500 mL. An aliquot of the sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 0.1 mg/kg, extract concentration is 0.10 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.005 to 1.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

The 500 µg/mL stock solution is also used to fortify accuracy and precision specimen.

Procedural recoveries were performed at 0.10 mg/kg, equivalent to 0.01 µg/mL.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

The method was found to be linear over the range 0.005 – 1.0 µg/mL for thymol. Eight calibration standards were quantified. The equation to the calibration line was $20589605x - 233875$ and the correlation coefficient $R = 0.9992$

Specificity:

Specificity was studied by analysis samples of standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol.

Interferences or contamination peak were not observed.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape samples, treated grape samples, untreated grape samples spiked with thymol for each ion monitored. Full mass spectrometry scan of thymol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The LOQ was defined as the lowest level for which acceptable recovery was demonstrated in study number AF/11125/ED (Bailey, 2008). LOQ is 0.01 mg/kg of thymol residue in sample. This level equates

to a calibration standard concentration of 0.01 µg/ml.

Accuracy, Repeatability (precision):

Accuracy was verified at one fortification level through procedural recoveries.

Fortification level (mg/kg)	0.1
Thymol recovery (%)	93
%RSD	9.2
n	8

Stability:

Stability in the matrix was investigated at 1 month, 3 months and 6 months (34, 93 and 185 days).

Results were as follows:

Time point (days)	0	34	93	185
Recovery (%)	98 (n = 3)	42 (n = 3)	38 (n = 3)	43 (n = 3)

Thymol is not stable for 1 month (34 days) in the matrix.

Conclusions

Thymol residues are extracted from grape sample with acetone. An aliquot is sampled, filtered over a 0.45 µm PTFE filter and quantified by GC-MS (three ions monitored).

The method described was validated by definition of the specificity, the linearity, the accuracy and the precision of the method as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission. Full mass spectrometry scan of thymol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification. The Limit of Quantification was 0.01 mg/kg for thymol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of thymol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 (2000) and SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/11125/ED (Bailey, 2008), which is included in this submission.

The method is acceptable for the quantification of thymol residues in grapes.

KCP 5.2/04 (A 2.3.1/06 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.2/04 (A 2.3.1/06 of this dRR)
Report author	Brown D.
Report year	2012
Report title	To determine the stability of thymol, eugenol and geraniol residues in grape specimens following storage at ca. -18° for 0, 1, 3, 7, 14 and 28 days, 3, 6 and 12 months after treatment with 3AEY (6.4% w/w geraniol, 3.2% w/w eugenol and 6.4% w/w thymol)
Report No	AD/12351/ED
Document No	Not applicable

Guidelines followed in study	Commission Directive 96/68/EC (amending Council Directive 91/414/EC) 7032/VI/95 rev.5 SANCO/3029/99 SANCO/825/00
Deviations from current test guideline	The method presented is in line with Guidance Document SANCO/825/00 rev.8.1
Previous evaluation	Partially evaluated (up to 28 days) and accepted in DAR (2011) under data point IIA 6.1.1/02
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples are homogenized and mixed with acetone. The mixed sample is dried over anhydrous sodium sulphate and centrifuged. An aliquot is sampled, dried over anhydrous sodium sulphate, filtered over a 0.45 µm PTFE filter and thymol residues are quantified by GC-MS.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

The method was validated prior to use and validation data is presented in Agrisearch study AF/10728/ED in the same test facility, which is included in this submission. However due to unavailability of the chromatographic column, a new chromatographic column and oven conditions were used. These changes are not expected to have an impact on the analytical method performance except for the retention time of the analytes.

Materials and methods

Test material

Test Standards

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	Fluka, lot n°1130656 Fluka, lot n°1305941
Active substance content:	99.9% 99.9%
Expiry date of lot/batch:	31 August 2007 31 August 2009
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with an HP6890 Autosampler and 5973N mass selective detector
Analytical column:	2B-Multiresidue-1, 30m x 0.25mm id (0.25µm film)
Oven:	50°C (2 min) – 10°C/min – 190°C (1 min) – 40°C/min – 330°C (10 min)
Detector temperature:	Transfer line, 285°C
Injector temperature:	250°C
Injection volume	Not stated
Injection mode	Splitless mode, temperature 250°C
Carrier gas:	Helium
Flow rate:	1 mL/min
Ionization mode	Selected ion monitoring
Retention time:	Thymol: Approx. 11.25 min
Monitored ions	135 m/z (quantification), 150, 115 m/z (qualification)

Sample preparation

Specimen preparation: Samples are sorted to remove stalks and homogenized with the aid of siphon carbon dioxide to maintain homogeneity.

Extraction: A 50 g subsample is transferred to a 250 mL nalgene jar and acetone (100 mL) is added. Fortification is performed at this point if necessary. The sample is homogenized for 30 seconds and anhydrous sodium sulphate is added. The sample is macerated for a further minute and centrifuged at 2500 rpm for 5 minutes. The sample is filtered over a 0.45 µm PTFE filter prior to quantification by GC-MS.

For samples fortified at 1.0 mg/kg, extract concentration is 0.5 µg/mL.

Stock solutions and calibration standards

A 500 µg/mL stock solution is prepared in ethyl acetate. This stock is sequentially diluted in acetone to produce calibration standards covering the range 0.005 to 1.0 µg/mL (corresponding to 0.01 to 2.0 mg/kg of residues). Matrix effects were not observed and hence calibration standards were prepared in solvent.

Recovery and precision samples

Specimen fortification was carried out using a 3AEY prepared in water.

Calculations

A calibration line of equation $y = ax + b$ is constructed using standard concentration and analyte peak area.

The residue is calculated using the bracketing standard method, by bracketing injections (typically not greater than 4) by a standard solution of known concentration. Quantitation is achieved by reference to the mean response of the appropriate bracketing standards as follows:

$$\text{Analyte residue (mg/kg)} = \frac{A \times B \times C \times F}{D \times E}$$

Where:

A = Peak area of relevant analyte in extract

B = Concentration of standard solution bracketing the analytical extract (µg/ml)

C = Volume of final extract (ml)

D = Weight of matrix in final volume of extract (g)

E = Average response factor of standard, e.g. $0.5 \times (H1 + H2)$

H1 = Peak area of the first bracketing standard solution injection

H2 = Peak area of the second bracketing standard solution injection

F = Extract dilution factor (if applicable)

Findings

The method described in this study was previously validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. Therefore, only partial validation data (linearity, procedural recoveries) and a single ion are presented to confirm the suitability of the method.

Linearity:

Linearity was investigated over the range 0.005 – 1.0 µg/mL. Five calibration standards were quantified. The equation to the calibration line was $1955827x - 21732$ and the correlation coefficient $R = 0.9998$. The linearity of the method is validated for this range of concentration.

Specificity:

Analysis of untreated control samples indicated a small level of interference from co-extracted material with residues ranging from 0.242 mg/kg at Day 0, 0.186 mg/kg at Day 1, 0.161 mg/kg at Day 3 and 0.098 mg/kg at Day 7. Thymol residues were <0.05 mg/kg for all the later timepoints. The level in the control samples was not consistent across time points but did appear to decrease over time. No residues of thymol were observed in control samples analysed after 14 days and therefore comparison of results from stored samples versus the nominal applied is considered to be reliable.

Copies of relevant chromatograms are provided for standards of thymol, untreated grape samples, treated grape samples, untreated and treated grape samples spiked with thymol for each ion monitored. Full mass spectrometry scan of thymol under the described analytical conditions was performed as part of study AF/10728/ED to confirm analyte identification.

LOD, LOQ:

The limit of quantitation is set at 0.05 mg/kg as validated as part of study number AF/10728/ED (Bailey, 2007). This level equates to a calibration standard concentration of 0.025 µg/ml.

Accuracy, Repeatability (precision):

Accuracy was verified at one fortification level through procedural recoveries at 1.0 mg/kg fortification.

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	80	85	82	75	86	90	86	96	76
Overall	Mean = 84%, RSD = 9.4%; n = 18								

Stability:

Stability of the thymol residues in the matrix was investigated at 0, 1, 3, 7, 14, 27, 94, 189 and 366 days).

Results were as follows:

Time point (days)	0	1	3	7	14	27	94	189	366
Mean recovery (%)	80	74	81	82	85	78	96	92	79
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)

Thymol is stable for up to one year in the matrix when stored frozen at -18°C.

Conclusions

Thymol residues are extracted from raw commodity grape samples with acetone, dried over anhydrous sodium sulphate and quantified by GC-MS (three ions monitored).

The method described was validated for specificity, linearity, accuracy and precision as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission. The Limit of Quantification was 0.05 mg/kg for thymol residues in grape samples.

This study was performed in the same test facility and confirmation of the suitability of the method is provided via linearity check and procedural recoveries. The method is confirmed as acceptable for the quantification of thymol residue in grapes.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol residues in grapes was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/825/00 rev.7 (2004) and was successfully validated as part of study number AF/10728/ED (Bailey, 2007), which is included in this submission.

The method is acceptable for the quantification of thymol residues in grapes.

Body fluids (Toxicology)

KCA 4.1.2/01 (A 2.3.1/07 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis for thymol in olive oil, 1% methyl cellulose in water, mouse plasma and rat plasma was previously evaluated at EU level.
-------------------	--

Data point:

CA 4.1.2/01 (A 2.3.1/07 of this dRR)

Report author

Brice A., Heslop D.

Report year	2009
Report title	Validation for the Determination of Thymol in Dietary Formulation, Mouse Plasma and Rat Plasma
Report No	8201847-D2149
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	Only two MS ions were followed for quantification and qualification. However, analyte identification is not in doubt and does not need confirmation since the analytical method validation is performed with analytical grade thymol using residue-free matrices. Two MS ions are sufficient to accurately identify and quantify thymol in the various matrices. Data is reported for one ion only. Mouse plasma lowest calibration standard was 10 ng/mL, equivalent to a sample fortification of 50 ng/mL. Rat plasma lowest calibration standard was 50 ng/mL which corresponds to a sample fortification of 250 ng/mL.
Previous evaluation	Already evaluated and accepted in DAR (2011) under data point IIA 6.1.1
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified in olive oil, 1% methylcellulose in water, mouse plasma and rat plasma using GC-MS. Various extraction techniques were used that are described below.

Materials and methods

Analytical grade thymol was used as test item

Test material

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Penta Manufacturing Company, batch n°103366,
Active substance content:	99.7% (equivalent to 997 g/kg)
Expiry date of lot/batch:	15 October 2010
Storage conditions:	Room temperature

Analysis parameters

	Olive oil, methyl cellulose, rat plasma, mouse plasma
Method type	GC-MS
Instrument:	Thermo Trace DSQ, CTC Analytics Combi Pal autosampler
Analytical column:	HP-5 30 m × 0.32 mm × 0.25 µm film thickness
Oven:	50°C initial temperature, hold for 0.5 minutes, ramp at 20°C/min to 270°C, hold for 0.5 minutes
Transfer line temperature:	270°C
Injector temperature:	230°C
Injection volume	1 µL
Injector inlet temperature:	230°C Split (10 mL/min flow)
Carrier gas:	Helium
Flow rate:	1 mL/min
Acquisition type:	SIM
Retention time:	Thymol: approx. 6.2 min Propofol (Internal standard): approx. 6.7 min
Monitored ions/transitions	Thymol: 135 and 150 m/z Propofol (Internal standard): 163 m/z

Sample preparation

Olive oil:

Control samples of olive oil were fortified with thymol at 20 and 200 mg/mL. Five separate fortifications were prepared at each level.

Dilution 1: 50 µL of fortified olive oil is transferred to a 25 mL volumetric flask. 1 mL of propofol internal standard solution (4 mg/mL) is added and the volume is adjusted with hexane to produce samples of concentrations 0.04 and 0.4 mg/mL from the 20 and 200 mg/mL fortification samples, respectively.

Dilution 2: Transfer 100 µL of the Dilution 1 samples to a fresh 1.5 mL vial and immediately add 900 µL of hexane to produce samples of concentrations 0.004 and 0.04 mg/mL from the 20 and 200 mg/mL fortifications, respectively.

Dilution 3: Transfer 100 µL of the Dilution 2 samples to a fresh 1.5 mL vial and immediately add 900 µL of hexane to produce samples of concentrations 0.4 and 4 µg/mL from the 20 and 200 mg/mL fortifications, respectively.

Dilution 4: Transfer 50 µL of the Dilution 3 samples to a fresh 1.5 mL vial and immediately add 950 µL of hexane to produce samples of concentrations 20 and 200 ng/mL from the 20 and 200 mg/mL fortifications, respectively.

Samples from Dilution 4 are subject to quantification via GC-MS.

1% methyl cellulose in water:

Control samples were fortified with thymol at 20 and 250 mg/mL. Five separate fortifications were prepared at each level.

20 mg or 250 mg are accurately weighed into a 20 mL amber glass vial. 1 mL of 1% methylcellulose in water is added immediately to produce solutions of concentrations 20 and 250 mg/mL respectively. 1 mL of 90 mg/mL propofol internal standard in ethanol is immediately added and the vial is capped.

Dilution 1: add 10 mL ethanol to the vial, cap and shake well before transferring to a 50 mL volumetric flask. Rinse the vial twice with 10 mL ethanol and transfer the rinsate to the volumetric flask. Adjust to the mark with ethanol to produce solutions of concentration 0.4 and 5.0 mg/mL from the 20 and 250 mg/mL fortifications, respectively.

Dilution 2: Transfer 100 µL of the Dilution 1 samples to a 20 mL volumetric flask and dilute to volume with hexane to produce samples of concentrations 0.002 and 0.025 mg/mL from the 20 and 250 mg/mL fortifications, respectively.

Dilution 3: Transfer 100 µL of the Dilution 2 samples into a fresh 1.5 mL glass vial already containing 900 µL of hexane, cap and mix well to produce samples of concentrations 0.2 and 2.5 µg/mL from the 20 and 250 mg/mL fortifications, respectively.

Dilution 4: Transfer 100 µL of the Dilution 3 samples into a fresh 1.5 mL glass vial already containing 900 µL of hexane, cap and mix well to produce samples of concentrations 20 and 250 ng/mL from the 20 and 250 mg/mL fortifications, respectively.

Samples from Dilution 4 are subject to quantification via GC-MS.

Mouse plasma:

Control samples were fortified with thymol at 10 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 500 ng/mL, 20 000 ng/mL and 35 000 ng/mL. Five separate fortifications were prepared at each level.

50 µL of sample are transferred to a 1.5 mL microcentrifuge tube and 200 µL of 50 ng/mL propofol internal standard in 1:1 v/v hexane:ethyl acetate is added. The sample is vortexed for about 30 seconds and micro centrifuged if necessary. The upper organic phase is transferred to a chromatography vial and quantified by GC-MS. For high concentration samples (or 20 000 and 35 000 ng/mL fortification), the samples are diluted 40-fold with control plasma prior to mixing with internal standard and analysis.

The extraction procedure yields the following extract concentrations: 2, 20, 40, 60, 100, 100 and 175 ng/mL.

Rat plasma

Control samples were fortified with thymol at 100 ng/mL 200 ng/mL, 300 ng/mL, 500 ng/mL and 20 000 ng/mL. Five separate fortifications were prepared at each level.

50 µL of sample are transferred to a 1.5 mL microcentrifuge tube and 200 µL of 50 ng/mL propofol internal standard in 1:1 v/v hexane: ethyl acetate is added. The sample is vortexed for about 30 seconds and micro centrifuged if necessary. The upper organic phase is transferred to a chromatography vial and

quantified by GC-MS. For high concentration samples (or 20 000 ng/mL fortifications), the samples are diluted 40-fold with control plasma prior to mixing with internal standard and analysis.

The extraction procedure yields the following extract concentrations: 20, 40, 60, 100, and 100 ng/mL.

Stock solutions and calibration standards

Internal standard (propofol):

Olive oil	Propofol is dissolved into hexane to produce a 4000 µg/mL internal standard solution. This internal standard solution should be stored frozen when not in use.
1% methyl cellulose in water	Propofol is dissolved in ethanol to produce a 90 mg/mL internal standard solution. This internal standard solution should be stored frozen when not in use. Dilute a 100 µL aliquot of the 90 mg/mL solution to 10 mL using a 10 mL volumetric flask with hexane (900 µg/mL). Further dilute a 50 µL aliquot of the 900 µg/mL into a 50 mL volumetric flask and dilute to volume with hexane to give a 900 ng/mL solution for use in preparing the calibration samples. This internal standard solution should be stored frozen when not in use.
Mouse plasma	Accurately weigh ca. 20 mg of Propofol into a 20 mL volumetric flask. Dilute to the mark using 1:1 v/v hexane:ethyl acetate to give internal standard of approximate concentration 1000 µg/mL.
Rat plasma	This solution is diluted sequentially using 1:1 v/v hexane:ethyl acetate to produce a final internal standard solution of concentration 50 ng/mL.

Calibration standards in olive oil:

A 250 mg/mL thymol stock solution in olive oil is prepared by weighing ca. 2500 mg thymol in a 10 mL volumetric flask and diluting to the mark with olive oil. The stock standard also acts as the highest calibration standard to be diluted. Calibration standards in olive oil are prepared through serial dilution. Standard solutions must be stored frozen in glass when not in use. A total of ten standards spanning the range 5 mg/mL to 250 mg/mL are prepared. Calibration standards are matrix-matched.

An aliquot of each calibration standard is combined with internal standard and diluted in hexane as per Dilution 1 and sequential dilutions 2, 3 and 4 are performed to produce a calibration range spanning the concentration range 5 ng/mL to 250 ng/mL.

Calibration standards in 1% methyl cellulose in water:

A 1000 µg/mL thymol stock solution in hexane is prepared by weighing 50 mg of thymol into a 50 mL volumetric flask, dissolving and adjusting to volume with hexane. Intermediate stocks are produced by serial dilution with hexane until a 1000 ng/mL stock is obtained. This 1000 ng/mL stock is diluted in hexane and internal standard solution (fixed 1000 µL volume) in order to produce a range of 12 calibration standards spanning the range 5 ng/mL to 300 ng/mL.

Calibration standards in rat and mouse plasma:

A 5000 µg/mL thymol stock solution is prepared by accurately weighing 50 mg of thymol into a 10 mL volumetric flask, dissolving in and adjusting to volume with methanol. This stock is diluted with methanol to produce an intermediate stock from which 10 calibration standards in methanol are produced, spanning a concentration range of 5 µg/mL to 75 µg/mL.

In order to produce matrix-matched calibration standards, 10 µL of each of the desired standards is mixed with 990 µL of control plasma. The final matrix-matched calibration range covers the concentrations 50 ng/mL to 750 ng/mL.

In addition, to support a LOQ of 10 ng/mL in mouse plasma, a supplementary matrix-matched standard in mouse plasma at a concentration of 10 ng/mL was prepared as described above.

Accuracy (recovery) samples

Olive oil:

To assess method performance, the method is validated at the following levels: 20 and 200 mg/mL thymol in olive oil. The 250 mg/mL prepared stock solution of thymol in olive oil is serially diluted using olive oil to prepare solutions at 20 and 200 mg/mL. The solutions are serially diluted as described in sample preparation above and analysed by GC-MS. Extract concentrations are 20 and 200 ng/mL from the 20 mg/mL and 200 mg/mL fortifications, respectively.

1% methyl cellulose in water:

To assess method performance, the method is validated at the following levels: 20 and 250 mg/mL thymol in 1% methyl cellulose in water. Thymol is directly weighed and diluted in 1 mL 1% methyl cellulose in water. Internal standard is added and the mixture is diluted as described in sample preparation above and analysed by GC-MS. Extract concentrations are 20 and 250 ng/mL from the 20 mg/mL and 250 mg/mL fortifications, respectively.

Rat and mouse plasma:

To assess method performance, the method is validated at the following levels: 100, 200, 300, 500 and 20 000 ng/mL. Further fortifications at 10 ng/mL and 35 000 ng/mL are prepared in mouse plasma only. The 5000 µg/mL thymol stock solution in methanol is used to prepare sequential accuracy dilutions spanning the concentration range 10 µg/mL to 2 000 µg/mL. Fortified samples are prepared by spiking 990 µL aliquots of plasma with 10 µL of the appropriate accuracy dilution. The solutions are combined with internal standard in 1:1 hexane:ethyl acetate v/v as described in sample preparation above and analysed by GC-MS.

Final extract concentrations are as follows:

Fortification (ng/mL)	10*	100	200	300	500	20 000	35 000*
Extract Conc. (ng/mL)	2	20	40	60	100	100	175

*: mouse plasma only

Calculations

Olive oil: Concentrations of thymol are determined by the interpolation of the peak area ratio of test article and the internal standard. The calibration line is determined by plotting the responses from the calibration solutions (y) against the amount of test substance injected (x) to generate a quadratic curve:

$$y = ax^2 + bx + c$$

Where a is the quadratic coefficient (curvature), b is the linear coefficient (gradient) and c is the intercept. The residue of thymol in each test sample is calculated as follows:

$$\text{Residue (mg/mL)} = \frac{\text{extract concentration (mg/mL)} \times \text{final volume (mL)}}{\text{sample volume (mL)}}$$

1% methyl cellulose in water: Concentrations of thymol are determined by the interpolation of the peak area ratio of test article and the internal standard. The calibration line is determined by plotting the responses from the calibration solutions (y) against the amount of test substance injected (x) to generate a quadratic curve:

$$y = ax^2 + bx + c$$

Where a is the quadratic coefficient (curvature), b is the linear coefficient (gradient) and c is the intercept. The residue of thymol in each test sample is calculated as follows:

$$\text{Residue (mg/mL)} = \frac{\text{extract concentration (mg/mL)} \times \text{final volume (mL)}}{\text{sample volume (mL)}}$$

Rat and mouse plasma: Concentrations of thymol are determined by the interpolation of the peak area ratio of test article and the internal standard. The calibration line is determined by plotting the responses from the calibration solutions (y) against the amount of test substance injected (x) to generate a quadratic curve:

$$y = ax^2 + bx + c$$

Where a is the quadratic coefficient (curvature), b is the linear coefficient (gradient) and c is the intercept. The residue of thymol in each test sample is calculated as follows:

$$\text{Residue (mg/mL)} = \frac{\text{extract concentration (mg/mL)} \times \text{final volume (mL)}}{\text{sample volume (mL)}}$$

Findings

Linearity:

Olive oil	Quadratic over the range 0 to 250 mg/mL. n = 6 Lowest calibration standard concentration: 5 ng/mL Equation to the calibration line: $y = -0.0079183 + 0.00545282x - 3.40051e-08x^2$ Coefficient of determination $R^2 = 0.9994$ Coefficient of correlation $r = 0.9997$
1% Methyl cellulose	Quadratic over the range 0 to 300 mg/mL. Lowest calibration standard concentration: 5 ng/mL Equation to the calibration line: $y = -0.0280835 + 0.00329798x + 4.19013e-06x^2$ Coefficient of determination $R^2 = 0.9971$ Coefficient of correlation $r = 0.9985$
Mouse plasma	Linear over the range 10 to 750 ng/mL Equation to the calibration line: $y = -0.0391321 + 0.00209x + 1.21701e-07x^2$ Coefficient of determination $R^2: 0.9988$ Coefficient of correlation $r = 0.9994$
Rat plasma	Linear over the range 50 to 750 ng/mL Equation to the calibration line: $y = -0.017802 + 0.00178817x + 5.09444e-07x^2$ Coefficient of determination $R^2: 0.9955$ Coefficient of correlation $r = 0.9977$

Specificity:

Olive oil	The ability of the method to distinguish between thymol and other substances present in the control samples was demonstrated. Components present in control samples that interfere with the analysis were not present at levels greater than 30% of the limit of quantification
1% Methyl cellulose	
Mouse plasma	Control extracts were free of any significant interference in the chromatographic regions of interest for the analyte.
Rat plasma	

Copies of relevant chromatograms are provided for control samples with internal standard for each matrix (olive oil, 1% methyl cellulose, mouse and rat plasma), and control samples spiked with thymol for each matrix (olive oil, 1% methyl cellulose, mouse and rat plasma).

LOD, LOQ:

Olive oil	Acceptable mean recovery (70 to 110%), and a relative standard deviation (RSD) of $\leq 20\%$ was obtained for the analysis of thymol in olive oil and 1% methyl cellulose at 20 mg/mL. Therefore, the LOQ was confirmed as 20 mg/mL for thymol.
1% Methyl cellulose	
Mouse plasma	Acceptable mean recovery (70 to 110%), and a relative standard deviation (RSD) of $\leq 20\%$ was obtained for the analysis of thymol in rat plasma at 10 ng/mL. Therefore, the LOQ was confirmed as 10 ng/mL for thymol.
Rat plasma	Acceptable mean recovery (70 to 110%), and a relative standard deviation (RSD) of $\leq 20\%$ was obtained for the analysis of thymol in rat plasma at 100 ng/mL. Therefore, the LOQ was confirmed as 100 ng/mL for thymol.

Accuracy, Repeatability (precision):

Matrix	Test Concentration	Extract concentration	Recovery (%)	RSD (%)	Horrat (RSD Horwitz value)
Olive oil	20 mg/mL:	20 ng/mL	90%	1.9%	0.79 (2.41)
	200 mg/mL:	200 ng/mL	103%	1.5%	0.88 (1.71)
	Combined:	-	97%	6.8%	-
1% Methyl cellulose	20 mg/mL:	20 ng/mL	93%	2.3%	0.95 (2.41)
	250 mg/mL:	250 ng/mL	104%	0.5%	0.29 (1.71)
	Combined:	-	99%	6.1%	-

Matrix	Test Concentration	Extract concentration	Recovery (%)	RSD (%)	Horrat (RSD Horwitz value)
Mouse plasma	10 ng/mL:	2 ng/mL	100.0%	4.0%	0.19 (21.44)
	100 ng/mL:	20 ng/mL	100.4%	3.2%	0.21 (15.16)
	200 ng/mL:	40 ng/mL	99.4%	2.8%	0.20 (13.66)
	300 ng/mL:	60 ng/mL	100.0%	2.4%	0.19 (12.85)
	500 ng/mL:	100 ng/mL	105.7%	1.4%	0.12 (11.90)
	20 000 ng/mL:	100 ng/mL	103.0%	1.6%	0.23 (6.83)
	35 000 ng/mL:	175 ng/mL	98.1%	1.9%	0.30 (6.28)
	Combined:	-	101.0%	2.55%	-
Rat plasma	100 ng/mL:	20 ng/mL	96.8%	5.0%	0.33 (15.16)
	200 ng/mL:	40 ng/mL	103.9%	3.5%	0.26 (13.66)
	300 ng/mL:	60 ng/mL	107.8%	2.2%	0.17 (12.85)
	500 ng/mL:	100 ng/mL	103.7%	3.5%	0.29 (11.90)
	20 000 ng/mL:	100 ng/mL	108.8%	1.7%	0.25 (6.83)
	Combined:	-	104.2%	1.3%	-

The data indicates that the accuracy and precision of the method is acceptable for all test matrices (olive oil, 1% methyl cellulose, mouse and rat plasma), in that the mean recoveries is well within the range (70 - 120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Olive oil	Stable for at least 56 days under frozen conditions
1% Methyl cellulose	Stable for at least 59 days under frozen conditions
Mouse plasma	Stable for at least 33 days under deep freeze conditions, for 24 hours at room temperature and through three freeze-thaw cycles
Rat plasma	Stable for at least 36 days under deep freeze conditions, for 24 hours at room temperature and through three freeze-thaw cycles

Conclusions

Thymol is extracted in olive oil, 1% methyl cellulose in water, in rat and mouse plasma with various extraction techniques, and then quantified by GC-MS (two ions monitored). Propofol was used as internal standard.

Thymol was successfully quantified in olive oil and 1% methyl cellulose in water and the methods proposed have been fully validated.

Thymol was also successfully quantified in rat and mouse plasma, however, validation with respect to linearity did not cover the totality of the analyte concentration range.

Specificity of the method and absence of interference from the test item, internal standard or the various matrices was demonstrated. Although data are available for a single ion, analyte identification is confirmed since the totality of the work was performed using blank matrices and analytical grade thymol. The ions followed are therefore confirmed to correspond to thymol. Moreover two ions were followed and all recoveries and relative standard deviations were within acceptable ranges, demonstrating the accuracy and precision of the various methods reported herewith.

The methods presented herewith are satisfactory and can be applied to quantify thymol olive oil and 1% methyl cellulose in water, as well as rat and mouse plasma, down to validated levels of 20 mg/mL, 20 mg/mL, 300 ng/mL and 100 ng/mL respectively (sample concentration).

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in olive oil, 1% methyl cellulose in water, mouse

plasma and rat plasma was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 and was successfully validated.
The method is acceptable for the quantification of thymol in olive oil, 1% methyl cellulose in water, mouse plasma and rat plasma down to validated levels of 20 mg/mL, 20 mg/mL, 100 ng/mL and 300 ng/mL respectively (sample concentration).

KCA 4.1.2/02 (A 2.3.1/08 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/02 (A 2.3.1/08 of this dRR)
Report author	XXXXXX
Report year	2009
Report title	Thymol: Oral (Gavage) Administration Pharmacokinetic Study in the Mouse
Report No	8202028
Document No	Not applicable
Guidelines followed in study	Not stated
Deviations from current test guideline	None. Analytical methods used in this study were validated within report n°8201847 (Brice & Heslop, 2009)
Previous evaluation	Already evaluated and accepted in DAR (2011) under data point IIA 6.1.1
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified in olive oil and mouse plasma using GC-MS.
The methods used in this study were fully validated within report n°8201847 (Brice & Heslop, 2009), which is submitted as part of this application. Please refer to report n°8201847 (Brice & Heslop, 2009, CA 4.1.2/1) for analytical methodology and validation data.

Assessment and conclusion by applicant:

The validation of the method was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 and was successfully validated as part of report n°8201847 (Brice & Heslop, 2009). The method is acceptable for the quantification of thymol in olive oil and mouse plasma.

KCA 4.1.2/03 (A 2.3.1/09 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/03 (A 2.3.1/09 of this dRR)
Report author	XXXXX
Report year	2009
Report title	Induction of chromosome aberrations in the bone marrow of treated rats
Report No	8201846
Document No	Not applicable
Guidelines followed in study	OECD 475 European Agency for the Evaluation of Medicinal Products. (1995). ICH Topic S 2 A. "Genotoxicity: Guidance on Specific Aspects of Genotoxicity Tests for Pharmaceuticals". ICH Harmonised Tripartite Guideline EC Commission Directive 2000/32/EC Annex 4C – B.12, Mutagenicity-In vivo Mammalian Erythrocyte Micronucleus Test No. L 136/50.
Deviations from current test guideline	None. Analytical methods used in this study were validated within report n°8201847 (Brice & Heslop, 2009)
Previous evaluation	Already evaluated and accepted in DAR (2011) under data point IIA 6.4.2
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified 1% methylcellulose in water and rat plasma using GC-MS.

The methods used in this study were fully validated within report n°8201847 (Brice & Heslop, 2009), which is submitted as part of this application. Please refer to report n°8201847 (Brice & Heslop, 2009) for analytical methodology and validation data.

Assessment and conclusion by applicant:

The validation of the method was previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 and was successfully validated as part of report n°8201847 (Brice & Heslop, 2009). The method is acceptable for the quantification of thymol in 1% methyl cellulose in water and rat plasma.

Avian diet (Ecotoxicology)

KCP 5.1.2/07 (A 2.3.1/10 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in avian diet was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/07 (A 2.3.1/10 of this dRR)
Report author	Martin K.H, Nixon W.B.
Report year	2007
Report title	Analytical method verification for the determination of 3 AEY

	(thymol/geraniol/eugenol mixture) in avian diet
Report No	648C-101
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and thymol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Thymol (reference material)
CAS number:	89-83-8

Source and lot/batch no.:	Sigma-Aldrich, lot n°60620
Active substance content:	100% w/w (equivalent to 1000 g/kg)
Expiry date of lot/batch:	August 2008
Storage conditions:	Ambient temperature

Analysis parameters

Method type	GC-FID
Instrument:	Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column:	DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven:	70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume:	2 µL
Injector: temperature:	250°C
Injection mode:	Splitless mode
Carrier gas:	Helium, CHP ≈ 28 PSI
Flow rate:	Hydrogen: 40 mL/min Air: 450 mL/min
Retention time:	Thymol: Approx. 7.2 min

Sample preparation

10 g of fortified diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) to fit within the calibration range:

- 3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 19.41 µg thymol⁸/mL
- 20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL), equivalent to 12.94 µg thymol⁷/mL

Calibration standards

A thymol stock solution was prepared by weighing 0.1000 g thymol analytical standard in a 100 mL volumetric flask and brought to volume with acetone. The concentration of the stock solution was 1000 µg/mL. This stock solution was used to prepare a combined secondary standard solution containing all three substances (eugenol, geraniol and thymol), although only thymol is considered in this summary. The secondary stock solution was prepared by transferring 10 mL of the primary stock solution into a 100 mL volumetric flask and adjusting to volume using 50:50 (v/v) acetone:hexane. The secondary stock concentration was 100 µg/mL. From this secondary stock solution, calibration standards spanning the concentration range 5.0 to 25.0 µg/mL in acetone/hexane 50:50 (v/v).

Accuracy and Recovery samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 194.1 ppm diet of thymol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 1423 ppm diet of thymol)

Calculations

The concentration of thymol found at the instrument was determined using the following equation:

$$\text{Thymol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

$$\text{Thymol (ppm)} = \left(\frac{\text{Thymol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity}$$

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 µg/mL. Five calibration standards were quantified. The equation to the calibration line was 34.689x + 2.7732 and the correlation coefficient R = 1.0000 (y = peak area of thymol, x = concentration of thymol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, reagent blank samples, matrix blank samples, and matrix samples spiked with thymol.

LOD, LOQ:

The Limit of Detection (LOD) was evaluated at the lowest analytical standard analyzed, and was observed to yield a detector response at least five times greater than the peak-to-peak background noise in the matrix blank extracts at the same dilution factor as the lowest matrix fortification.

ppm thymol equivalent

$$= \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}}$$

The Limit of Quantification (LOQ) was set at 3000 ppm of test item based upon the lowest matrix

⁸ Based on a thymol content of 6.47% w/w

fortification level analyzed concurrently with the samples, corresponding to 194.1 ppm (194.1 µg/g of diet).

Accuracy, Repeatability (precision):

Diet concentration (ppm)	Thymol concentration in diet (ppm)	Measured Thymol in diet (ppm)	% nominal	Mean	SD	RSD
3000	194.1	191	98.4	92.0	6.4	6.9
		174	89.6			
		164	84.5			
		192	98.9			
		172	88.6			
20000	1423	1064	74.8	84.4	6.9	8.1
		1273	89.5			
		1173	82.4			
		1314	92.3			
		1178	82.8			

The data indicates that the accuracy and precision of the method is acceptable at a 194.1 ppm and above in that the mean recoveries are well within the range (70 -110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set 194.19 ppm (194.1 µg/g of diet).

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and thymol is quantified by GC-FID. The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in avian diet. The Limit of Quantification 194.1 µg thymol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.
The method is acceptable for the quantification of thymol in avian diet.

KCP 5.1.2/08 (A 2.3.1/11 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in avian diet was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/08 (A 2.3.1/11 of this dRR)
Report author	XXXXXXXXXX
Report year	2007
Report title	3AEY (Thymol/Geraniol/Eugenol Mixture): a dietary LC50 study with the northern bobwhite
Report No	648-102
Document No	Not applicable
Guidelines followed in study	OECD 205

	EPA OPPTS 850.2200
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of test diet were extracted using acetone:hexane 50:50 v/v and thymol is quantified by GC-FID. Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no:	Eden Research plc, lot n°YP0A3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23% w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Analytical standard

Name:	Thymol (reference material)
Source and lot/batch no:	Sigma-Aldrich, lot n°60620
Active substance content:	100% w/w (equivalent to 1000 g/kg)
Expiry date of lot/batch:	August 2008
Storage conditions:	Ambient temperature

Analysis parameters

Method type	GC-FID
Instrument:	Hewlett-Packard Model 6890 Gas Chromatograph with flame ionization detector
Analytical column:	DB-5ms (30m x 0.25 mm I.D., 0.25 µm film thickness)
Oven:	70°C (1 min) – 10°C/min – 200°C (0 min) – 40°C/min – 280°C (1 min)
Injection volume:	2 µL
Injector temperature:	250°C
Injection mode:	Splitless mode
Carrier gas:	Helium, CHP ≈ 28 PSI
Flow rate:	Hydrogen: 40 mL/min Air: 450 mL/min
Retention time:	Thymol: Approx. 7.27 min

Sample preparation

Samples of the test diets were collected to verify the test concentrations administered and to confirm the stability and homogeneity of the test substance in the diets.

10 g of diet is combined with 100 mL acetone/hexane (50:50, v/v) and sonicated for 60 minutes. The sample is then shaken for 60 minutes at 300 rpm. A 20 mL aliquot is transferred to a scintillation vial and

centrifuged for 10 minutes at 1500 rpm.

Samples are diluted in acetone/hexane (50:50, v/v) depending on their nominal concentration:

- 3000 ppm: no dilution (extract concentration: 0.30 mg 3AEY/mL), equivalent to 19.41 µg thymol/mL
- 5000 ppm: 1/2 (extract concentration: 0.25 mg 3AEY/mL), equivalent to 16.175 µg thymol⁷/mL
- 10 000 ppm: 1/5 (extract concentration: 0.20 mg 3AEY/mL), equivalent to 12.94 µg thymol⁷/mL
- 20 000 ppm: 1/10 (extract concentration: 0.20 mg 3AEY/mL), equivalent to 12.94 µg thymol⁷/mL

Calibration standards

Calibration standards in the range 5 – 25 µg/mL are prepared in acetone/hexane 50:50 (v:v).

Recovery and precision samples

Two diet fortifications are prepared:

- 0.3 g test material/100 g of diet or 3000 ppm diet (corresponding to 194.1 ppm diet of thymol)
- 2.2 g test material/100 g of diet or 22 000 ppm diet (corresponding to 1423 ppm diet of thymol)

Diet homogeneity

Homogeneity of the test substance in the diet was evaluated by collecting six samples from the 5000 and 20000 ppm test diets at the time of preparation on Day 0 and Day 1. Homogeneity samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. The homogeneity samples also served as verification samples for those concentrations.

Calculations

The concentration of thymol found at the instrument was determined using the following equation:

$$\text{Thymol } (\mu\text{g/mL}) = \frac{\text{Peak area response} - \text{intercept}}{\text{Slope}}$$

The concentration expressed as ppm for each sample was determined using the following equation:

$$\begin{aligned} \text{Thymol (ppm)} &= \left(\frac{\text{Thymol } (\mu\text{g/mL}) \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{initial weight (g)}} \right) / \text{Purity} \\ \text{ppm thymol equivalent} &= \frac{\text{Lowest standard concentration } (\mu\text{g/mL}) \times \text{final vol. (mL)} \times \text{dilution factor}}{\text{Blank diet weight}} \end{aligned}$$

Fortification Recoveries

The ppm found in each sample is divided by the nominal concentration of each sample (fortified level, ppm) and multiplied by 100.

Findings

Linearity:

Linearity was investigated over the range 5.0 – 25 µg/mL. Five calibration standards were quantified. The equation to the calibration line was 35.1618x – 1.19498 and the correlation coefficient R = 0.9999 (y = peak area of thymol, x = concentration of thymol (in µg/mL)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, matrix blank samples, and matrix samples spiked with thymol.

LOD, LOQ:

The Limit of Detection (LOD) was set based upon the injection volume (2.00 µL) and the lowest standard concentration 5.00 µg a.i./mL. The LOD was set at 10.0 ng on-column.

The Limit of Quantification (LOQ) was set at 3000 ppm based upon the lowest matrix fortification level analyzed concurrently with the samples and the LOQ validated during the study 648-101C in the same test facility. The 5.00 µg a.i./mL thymol standard was equivalent to a calculated value of 50 ppm a.i. in

the matrix blank extract. Measured values greater than or equal to the ppm a.i. equivalent were reported for each analyte.

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 3000 ppm (194.1 ppm diet of thymol) and 22 000 ppm (1423 ppm diet of thymol).

Fortification level (mg/L)	3000 ppm	22 000 ppm	mean	%RSD	n
Accuracy Day 1	80	71	76	-	2
Accuracy Day 2	84	74	79	-	2
Overall			77.3	7.6	4

The results obtained confirmed the accuracy and the precision of the method at 3000 ppm (194.1 ppm diet of thymol) and above.

Homogeneity:

Diet concentration (ppm)	Thymol concentration in diet (ppm)		Measured Thymol in diet (ppm)	%nominal	Mean	SD	RSD
5000	323.5	Top left	333	102.94	82.5	11.4	13.8
		Top right	280	86.55			
		Middle left	237	73.26			
		Middle right	254	78.52			
		Bottom left	233	72.02			
		Bottom right	264	81.61			
20000	1294	Top left	1031	79.68	80.1	1.4	1.7
		Top right	1039	80.29			
		Middle left	1006	77.74			
		Middle right	1036	80.06			
		Bottom left	1056	81.61			
		Bottom right	1052	81.30			

The diet was homogenous.

Conclusions

Samples of test diet were extracted using acetone:hexane 50:50 v/v and thymol is quantified by GC-FID. The method described was validated by definition of specificity, linearity, accuracy and precision of the method as part of study number 648-101C (Martin, Nixon, 2007), which is included in this submission. The results obtained in this study confirmed that the method is linear, accurate and precise and suitable for the quantification of thymol in avian diet. The Limit of Quantification 194.1 µg thymol/g of avian diet.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in avian diet was not previously evaluated at EU level, although the dietary toxicity study on the Northern Bobwhite was. The study was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated as part of study number 648-101C (Martin, Nixon, 2007) at concentration levels relevant to the test's results. The method is acceptable for the quantification of thymol in avian diet.

Water (Ecotoxicology)

KCA 4.1.2/18 (A 2.3.1/12 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/18 (A 2.3.1/12 of this dRR)
Report author	XXXX
Report year	2008g
Report title	Acute Toxicity of THYMOL CRYSTALS to Rainbow Trout (Oncorhynchus mykiss) in a 96-hour Semi-static Test
Report No	34281230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Thymol crystals (technical grade)
CAS number:	89-83-8
Source and lot/batch no.:	Eden Research plc, lot n°94747
Active substance content:	99.7% w/w (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 15 min

Monitored ions/transitions

Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 500 mg test item into 5.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the test fish at the start of the test and just before the test medium renewal.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg dimethylformamide (DMF) /L 50% / 50% to obtain standard solutions in the range from 0.2 to 6 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 0.5, 1, 2 and 10 mg test item/L.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.2 – 6 mg/L. Seven calibration standards were quantified. The equation to the calibration line was $269714x - 98003$ and the correlation coefficient $R = 0.9976$ (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control solvent samples, control water samples, and water samples spiked with thymol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.05 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 1 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 0.5, 1.0, 2.0 and 10.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
0.5	170*	3	6
1.0	107	2	4
2.0	91	6	6
10.	97	4	6
Overall	93*	6*	16*

*The accuracy test failed at 0.5 mg/L (recovery > 110%). Therefore, the results at 0.5 mg/L is not included in overall accuracy/repeatability results.

The method is accurate and precise at a level of 1.0 mg/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 1.0 mg/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is not stable in the test medium. Therefore, results are expressed based on both nominal and actual concentrations.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding for the 96 hours test period). Additionally, at any observation date where all test fish were found to be dead at one test concentration, samples were taken from this concentration. After 48 hours of exposure the mean measured test item concentrations were 76.5% (63 - 87%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 77% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in fish test medium. The Limit of Quantification was 1.0 mg/L for thymol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in fish test medium.

KCA 4.1.2/19 (A 2.3.1/13 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CA 4.1.2/19 (A 2.3.1/13 of this dRR)
Report author	XXXXXX
Report year	2008h
Report title	Acute Toxicity of THYMOL CRYSTALS to Zebra Fish (Danio rerio) in a 96-hour Semi-static Test
Report No	34282230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.2.1.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Thymol crystals (technical grade)
CAS number:	89-83-8
Source and lot/batch no.:	Eden Research plc, lot n°94747
Active substance content:	99.7% w/w (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode

Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 15 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 400 g/L was prepared by dissolving 4000 mg test item into 10.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 ml/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to fish. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations. The test media were prepared just before introduction of the test fish at the start of the test and just before the test medium renewal.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg dimethylformamide (DMF) /L 50% / 50% to obtain standard solutions in the range from 0.5 to 6 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 2, 10 and 40 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.5 – 6 mg/L. Six calibration standards were quantified. The equation to the calibration line was $367079x - 127568$ and the correlation coefficient $R = 0.9982$ (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control solvent samples, control water samples, and water samples spiked with thymol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.02 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 2 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at three levels: 2.0, 10.0 and 40.0 mg/L:

Fortification level (mg/L)	Accuracy	%RSD	n
2.0	103	3	6
10.0	97	1	6
40.0	98	3	4
Overall	99	4	16

The method is accurate and precise at a level of 2.0 mg/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 2.0 mg/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium. Therefore, results are expressed based on nominal concentrations.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding to the 96 hours test period). Additionally, at any observation date where all test fish were found to be dead at one test concentration, samples were taken from this concentration. After 48 hours of exposure the mean measured test item concentrations were 91% (86 - 97%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 93% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in fish test medium. The Limit of Quantification was 2.0 mg/L for thymol in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in fish test medium.

KCA 4.1.2/20 (A 2.3.1/14 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in daphnia test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/20 (A 2.3.1/14 of this dRR)
Report author	Grade R., Wydra V.
Report year	2007b
Report title	Acute Toxicity of THYMOL CRYSTALS to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	34283220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202 EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.3.1.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

Materials and methods

Test material

Test Item

Name:	Thymol crystals (technical grade)
CAS number:	89-83-8
Source and lot/batch no.:	Eden Research plc, lot n°94747
Active substance content:	99.7% w/w (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode

Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 16 min
Monitored ions/transitions	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 1000 mg test item into 10.0 mL dimethylformamide (DMF) at test start and before the test medium renewal after 48 hours. These stock solutions were diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 ml/L) were added at each test concentration. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to daphnia. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations. The test media were prepared just before introduction of the test organism at the start of the test.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg DMF /L 50% / 50% to obtain standard solutions in the range from 0.2 to 6 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/100 mg DMF/L to obtain fortified samples at a level of 0.5, 1, 2 and 10 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.2 – 6 mg/L. Seven calibration standards were quantified. The equation to the calibration line was $337684x - 99603$ and the correlation coefficient $R = 0.9986$ (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control solvent samples, control water samples, and water samples spiked with thymol.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.02 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained.

The LOQ was found at 1 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 0.5, 1.0, 2.0 and 10.0 mg/L

Fortification level (mg/L)	Accuracy	%RSD	n
0.5	138*	11.7	4
1.0	107	2.4	4
2.0	95	1.8	4
10.0	99	2.4	4
Overall	100*	5.8*	12*

*The accuracy test failed at 0.5 mg/L (recovery > 110%). Therefore, the results at 0.5 mg/L is not included in overall accuracy/repeatability results.

The method is accurate and precise at a level of 1.0 mg/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 1.0 mg/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium. Therefore, results are expressed based on nominal concentrations.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after the end of 48 hours test period) by pouring together the contents of the test beakers of each treatment. After 48 hours of exposure the mean measured test item concentrations were 92.3% (86 - 98%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 98% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in daphnia test medium. The Limit of Quantification was 1.0 mg/L for thymol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in daphnia test medium.

KCA 4.1.2/21 (A 2.3.1/15 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of thymol in daphnia test medium was not previously evaluated at EU level.</p> <p>The method for determination of thymol in aqueous test medium used for an aquatic toxicity test has been validated according to the guideline SANCO/3029/99 rev.4.</p> <p>Final analysis: GC with MS detection</p> <p>Limit of quantification: LOQ was 0.0280 mg/L in aqueous test medium</p> <p>Limit of detection: LOD was defined as 30% of the LOQ (i.e. 0.0084 mg/L)</p> <p>The mean recovery at each fortification level was in the range of 70 – 110%. The relative standard deviation (RSD) calculated for the recovery data at each fortification level was below $\leq 20\%$.</p> <p>The method is acceptable for the quantification of thymol in daphnia test medium.</p>
-------------------	--

Data point:	CA 4.1.2/21 (A 2.3.1/15 of this dRR)
Report author	Egeler P
Report year	2021
Report title	A Study on the Chronic Toxicity to Daphnia magna [Analytical phase by Schrag K., 2021, Phase ID 20E13109-01-RADW]
Report No	20GC2DB
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev. 4
Deviations from current test guideline	None
Previous evaluation	No, not already evaluated
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are extracted in toluene and quantified by GC-MS.

Materials and methods

Reference item

Test Item

Name:	Thymol
CAS number:	89-83-8
Source and lot/batch no.:	ECT, Germany, lot n°THY/02/2019-20
Active substance content:	99.58% w/w (corresponding to 995.8 g/kg)
Expiry date of lot/batch:	31 July 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent GC 6890 with MS 5973 detector in EI mode
Analytical column:	Agilent VF-WAXms, 30 m x 0.32 mm i.d., 0.25 µm film thickness; Part No CP 9212
Oven:	100 °C initial, hold 3 min, 40 °C/min to 240 °C, hold 5 min
Injection volume:	2 µL
Injection mode:	Splitless mode
Injection temperature:	240 °C
Carrier gas:	Helium

Flow rate:	1.2 mL/min
Transfer Line temp.:	240 °C
MS Quad temp.:	150 °C
MS Source temp.:	230 °C
Retention time:	Approx. 6.9 min
Monitored ions/transitions	135 m/z, 150 m/z, 115 m/z (30 ms dwell time)

Sample preparation

The test solution specimens were taken out of the freezer storage. Before defrosting, formic acid (1% based on the sample volume; e.g. 0.2 mL to a water sample of 20 mL) and toluene (10:1 (v/v) based on the sample volume; e.g. 2 mL to a water sample of 20 mL) were pipetted into the sample vessels. The samples were then defrosted and homogenised for at least 2 min on a vortex mixer. After phase separation an aliquot of the upper organic phase was transferred into GC vials and used directly for analysis by GC-MS. Final samples were diluted in toluene to achieve final concentrations falling within the range of the calibration curve.

Stock solutions and calibration standards

Stock solution: a stock solution containing 1000 mg/L (S1000) of thymol was prepared by pipetting 25.15 µL of the reference item into a 25 mL volumetric flask and adjusting the volume to 25 mL with toluene.

Fortification solutions: From this stock solution, fortification solutions of 100 mg/L (S100), 10 mg/L (S10) and 1 mg/L (S1) were prepared in toluene.

Calibration standards: Chromatographic external standard solutions were prepared by diluting the 1 mg/L stock of the reference item with toluene. The calibration range included six standards spanning the range 42 – 1000 µg/L (corresponding to 0.0042 to 0.100 mg/L in the sample).

Fortification samples

Control samples of aqueous test medium were fortified with fortification solutions in order to produce fortified samples at 0.0280 and 2.20 mg/L. Control samples were treated as described for the samples.

Calculations

External standard solutions comparable to the concentration expected in specimens were injected before and after a maximum of 4 samples in the analytical sequence. The concentrations were directly calculated from the peak areas of the samples, using the mean peak area of the two bracketing standards as a one-point-calibration.

The concentration of thymol in the aqueous test samples in mg/L was calculated as follows:

$$C = \frac{c \times V_{EX} \times d}{V_w \times 1000}$$

Where:

C	Concentration of thymol in aqueous test specimens in mg/L
c	Concentration of thymol in final sample extracts, corrected with interspersed standards in µg/L
V _{EX}	Volume of extraction solvent (toluene, 2 mL)
d:	Dilution factor
V _w	Volume of water sample (20 mL)
1000	Conversion factor µg to mg

Recoveries were calculated by the following equation:

$$Rec = \frac{R_{found}}{R_{fortified}} \times 100\%$$

Where:

Rec	Recovery in %
R _{found}	Analyte determined in mg/L
R _{fortified}	Fortification level in mg/L

Findings

Linearity:

Linearity was investigated over the range 42 – 1000 µg/L. Six calibration standards were quantified.

	135 m/z	150 m/z	115 m/z
Equation	$y = 854.32x + 9558.07$	$y = 272.08x + 2645.38$	$y = 115.23x + 1760.63$
R ²	0.99978	0.99990	0.99986

The linearity of the method is validated for this range of concentration.

Specificity:

The concentration of thymol in the samples was determined by gas chromatography with MS detection using at least three characteristic mass fragments. Mass fragment m/z = 135 was used for quantification, mass fragments m/z = 150 and m/z = 115 were used for confirmation. No significant interferences from the test medium were detected at the retention time corresponding to the analyte in any of the control specimens.

Copies of relevant chromatograms are provided in the report for standards of thymol, control solvent samples, control water samples, and water samples spiked with thymol.

LOD, LOQ:

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 0.0280 mg/L.

The limit of detection (LOD) was defined as 30% of the limit of quantification. The LOD was found at 0.0084 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at two levels: 0.0280 and 2.20 mg/L:

Fortification level [mg/L]	Recoveries			No of analyses	Overall recovery		Overall recovery	
	Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]	Mean [%]	RSD [%]
Thymol quantifier mass fragment m/z = 135							97	4.0
0.0280	94, 95, 104, 104, 94	98	5.4	5	97	4.0		
2.20	95, 95, 98, 99, 96	97	1.9	5				
Thymol qualifier 1 mass fragment m/z = 150								
0.0280	94, 95, 103, 105, 94	98	5.5	5	97	4.0		
2.20	95, 95, 98, 99, 96	97	1.9	5				
Thymol qualifier 2 mass fragment m/z = 115								
0.0280	91, 96, 103, 101, 92	97	5.5	5	96	3.9		
2.20	94, 95, 97, 99, 96	96	2.0	5				

Mean recovery values obtained by GC-MS for thymol for all fortification levels comply with the standard acceptance criteria of guideline SANCO/3029/99 rev. 4, which demands that the mean recovery at each fortification level should be in the range of 70 - 110%. It is therefore concluded, that the method is suitable for aqueous test medium using GC with MS detection.

Moreover, all corresponding relative standard deviations of less than 20% indicate that the method demonstrates good precision and repeatability for aqueous test medium at the validated levels.

Procedural recoveries:

The aqueous test medium samples C2, C3 and C4 (reserve samples according to the study plan) were additionally analysed. Therefore, procedural recoveries, one at the LOQ of 0.0280 mg/L, one at 2.20 mg/L and one control sample were extracted and analysed along with the samples to prove the performance of the method.

Matrix	Fortification Level [mg/L]	Procedural Recovery [%]
Thymol quantifier mass fragment m/z = 135		
Aqueous test medium (provided by test facility)	0.0280	95
	2.20	97
Thymol qualifier 1 mass fragment m/z = 150		

Matrix	Fortification Level [mg/L]	Procedural Recovery [%]
Aqueous test medium (provided by test facility)	0.0280	95
	2.20	98
Thymol qualifier 2 mass fragment m/z = 115		
Aqueous test medium (provided by test facility)	0.0280	96
	2.20	95

With recoveries ranging from 70-110%, the method performance for the day of extraction of the reserve samples is proven.

Stability:

All sample extracts were analysed within 24 hours after extraction of thymol. Therefore, no stability testing is required.

Conclusions

Sample of aqueous test medium are extracted in toluene and quantified by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in daphnia test medium. The Limit of Quantification was 0.0280 mg/L mg/L for thymol in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in daphnia test medium was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in daphnia test medium.

KCA 4.1.2/22 (A 2.3.1/16 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in algal test medium was previously evaluated at EU level.
-------------------	--

Data point:	CA 4.1.2/22 (A 2.3.1/16 of this dRR)
Report author	Grade R., Wydra V. Hoffmann K, Wydra V. (amended report)
Report year	2008c, 2011 (amended report)
Report title	Toxicity of THYMOL CRYSTALS to <i>Pseudokirchneriella subcapitata</i> in an Algal growth Inhibition Test
Report No	34284210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade test item supported by a GLP certificate of analysis and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.

Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIA 8.4/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The samples were diluted with acetonitrile or acetonitrile / test medium (containing 100 mg DMF/L) 50% / 50%, if necessary, to fit within the calibration range prior to analysis of thymol content by GC-MS.

Materials and methods

Test material

Name:	Thymol crystals (technical grade)
CAS number:	89-83-8
Source and lot/batch no.:	Eden Research plc, lot n°94747
Active substance content:	99.7% w/w (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Dark, room temperature (+10°C to +30°C)

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (0 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume	1 µL
Injection mode	Splitless mode
Carrier gas:	Helium
Flow rate:	1 mL/min
Retention time:	Approx. 15 min
Monitored ions/transitions	Not stated

Sample preparation

The samples were diluted with acetonitrile or acetonitrile / test medium (containing 100 mg DMF/L) 50% / 50%, if necessary, to fit within the calibration range prior to quantification.

Stock solutions and calibration standards

Test item solutions: A concentrated stock solution of nominal 100 g/L was prepared by dissolving 2000 mg test item into 2.0 mL dimethylformamide (DMF). This stock solution was diluted in a series of sequential dilutions with the solvent to ensure that the same volumes of solvent (0.1 mL/L) were added in each test solution. The solvent additive was chosen based upon its solubility properties and its relative non toxicity to algae. Adequate volumes of these series of dilutions were mixed into test water to obtain the desired test concentrations.

The test media were prepared just before introduction of the algae at the start of the test.

Calibration standards: The test item was used to prepare a stock solution. 50 mg of test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L.

Appropriate amounts of the stock solution were diluted with a mixture of acetonitrile /test medium + 100 mg DMF /L 50% / 50% to obtain standard solutions in the range from 0.3 to 10 mg test item/L.

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL acetonitrile to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water/ 100 mg DMF/L to obtain fortified samples at a level of 0.8, 3, 10 and 100 mg test item/L. Exact values were documented in the raw data.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

% of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.3 – 10 mg/L. Seven calibration standards were quantified. The equation to the calibration line was $786175x - 201613$ and the correlation coefficient $R = 0.99788$ (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control solvent samples, control water samples, and water samples spiked with thymol.

LOD, LOQ:

The Limit of Detection is determined mathematically from the linear calibration curve according to DIN 32 645. The LOD was found at 0.779 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 3.2 mg/L.

Accuracy, Repeatability (precision):

Accuracy was verified at five levels: 1.0, 3.2, 10, 32 and 100 mg/L

Fortification level (mg/L)	Accuracy		%RSD		n	
Report/Amendment date	2008	2011	2008	2011	2008	2011
1.0	n.c. ¹	82 ²	-- ¹	14 ²	4 ¹	16 ²
3.2	85	84	16	16	4	4
10	82	81	16	16	4	4
32	85	84	17	17	4	4
100	79	79	9	9	4	4
Overall	83 ¹	82 ²	14 ¹	14 ²	16 ¹	16 ²

¹: not calculated. The accuracy test failed at 1.0 mg/L (results either below the LOD or below the LOQ). Therefore, the results at 1.0 mg/L was not included in overall accuracy/repeatability results in the original 2008 report.

²: Overall geometric mean value of test media at 3.2 to 100 mg/L (see discussion below)

The method is accurate and precise at a level of 3.2 mg/L and above in that the mean recoveries are well

within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 3.2 mg/L.

The test concentration of 1 mg test item/L could not be quantified with the used analytical method. Therefore, the overall geometric mean value was assumed as concentration for this treatment level. Since the geometric mean values in the treatment levels of 3.2 to 100 mg test item/L were all close 80 %, this was considered to be justified. This step was necessary, since for the re-evaluation of the statistical data a concentration has to be introduced for this treatment level.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls (containing algae) were taken at the end of the test (after the 96 hours test period). After 96 hours of exposure the mean measured test item concentrations were 72.6% (69 - 75%) of the nominal values. Thus, during the test period of 96 hours the algae were exposed to mean measured concentrations of 82% of nominal. All reported results in the report amendment are expressed in terms of the geometric mean concentrations of the test item.

Conclusions

The samples were diluted with acetonitrile or acetonitrile / test medium (containing 100 mg DMF/L) 50% / 50%, if necessary, to fit within the calibration range prior to analysis of thymol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in algal test medium. The Limit of Quantification was 3.2 mg/L for thymol in algal test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in algal test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in algal test medium.

KCP 5.1.2/09 (A 2.3.1/17 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/09 (A 2.3.1/17 of this dRR)
Report author	XXXXX
Report year	2008a
Report title	Acute Toxicity of 3AEY to Rainbow Trout (<i>Oncorhynchus mykiss</i>) in a 96-hour Semi-static Test
Report No	34301230
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.1 OECD 203 EPA OPPTS 850.1075
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference

	item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.1/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Thymol crystals (reference material)
CAS number:	89-83-8
Source and lot/batch no.	Sigma-Aldrich, lot n°94747
Active substance content:	99.7% (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	not stated

Retention time:	Thymol: Approx. 14.5 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to thymol, this is equivalent to 0.40, 0.81, 1.62, 3.24 and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w in the test item.

Stock solutions and calibration standards

Thymol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in

acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only thymol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 8 mg thymol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L and stirred to achieve solubilisation. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 15, 20 and 100 mg test item/L. With respect to thymol, this is equivalent to 0.32, 0.97, 1.29 and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation

$$\% \text{ of nominal} = (c/c_{\text{nom}}) \times 100\%$$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.1 – 3 mg/L and also over the range 0.8 to 8 mg/L.

Six calibration standards were quantified for the low-range linearity verification while five standards were included in the high-range linearity verification. The equations to the calibration lines were $976573x - 54245$ for low-range linearity and $1140605x - 220528$ for high-range linearity, the correlation coefficient R was at least 0.9963 (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.03 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 5 mg test item/L, corresponding to 0.332 mg thymol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 5.0, 15.0, 20.0 and 100 mg/L, corresponding to 0.32, 0.97, 1.29 and 6.47 mg thymol/L respectively,

Test item fortification level (mg/L)	Accuracy	%RSD	n
--------------------------------------	----------	------	---

5	93	3.4	4
15	100	6.5	4
20	88	6.3	4
100	105	8.7	6
Overall	97	9	18

The data indicates that the accuracy and precision of the method is acceptable at a 5.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 5.0 mg test item/L or 0.332 mg thymol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken just before the test medium renewal (after 48 hours) and at the end of the test (after the 48 hours of test medium renewal corresponding to the 96 hours test period). After 48 hours of exposure the mean measured test item concentrations were 95% (73 - 115%) of the nominal values. Thus, during the test period of 96 hours the fishes were exposed to mean measured concentrations of 96% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in fish test medium. The Limit of Quantification was 0.332 mg thymol/L in fish test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in fish test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in fish test medium.

KCP 5.1.2/10 (A 2.3.1/18 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in daphnia test medium was previously evaluated at EU level.
-------------------	--

Data point:	CP 5.1.2/10 (A 2.3.1/18 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008b
Report title	Acute Toxicity of 3AEY to <i>Daphnia magna</i> in a Static 48-hour Immobilization Test
Report No	34302220
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.2 OECD 202

	EPA OPPTS 850.1010
Deviations from current test guideline	No information on the daughter ions monitored is presented in the report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.2/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Thymol crystals (reference material)
CAS number:	89-83-8
Source and lot/batch no.:	Sigma-Aldrich, lot n°94747
Active substance content:	99.7% (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	Not stated
Retention time:	Thymol: Approx. 15 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 6.25, 12.5, 25.0, 50.0 and 100 mg/L. With respect to thymol, this is equivalent to 0.40, 0.81, 1.62, 3.24 and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w in the test item.

Stock solutions and calibration standards

Thymol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only thymol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.1 to 8 mg thymol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L and stirred to achieve dissolution. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 5, 10, 20 and 100 mg test item/L. With respect to thymol, this is equivalent to 0.32, 0.65, 1.29 and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w in the test item.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.1 – 8 mg/L.

Eight calibration standards were quantified. The equation to the calibration line was $1259556x - 280819$, the correlation coefficient R was 0.9980 (y = peak area of thymol, x = concentration of thymol (in mg/L)). The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for thymol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.01 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 20 mg test item/L, corresponding to 1.33 mg thymol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 5.0, 10.0, 20.0 and 100 mg/L, corresponding to 0.32, 0.65, 1.33 and 6.47 mg thymol/L respectively,

Test item fortification level (mg/L)	Accuracy	%RSD	n
5	180*	1.1	4
10	126*	6.9	4

20	95	3.0	4
100	90	4.3	4
Overall	97	5	8

*Accuracy was not acceptable at 5 and 10 mg/L. These values were not included in the overall calculations.

The data indicates that the accuracy and precision of the method is acceptable at a 20.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 20.0 mg test item/L or 1.33 mg thymol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated; however, analysis of 48 hours-old test medium demonstrated that the test item is stable in the test medium.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls were taken at the end of the test (after the end of 48 hours test period). After 48 hours of exposure the mean measured test item concentrations were 89% (86 - 92%) of the nominal values. Thus, during the test period of 48 hours the daphnia were exposed to mean measured concentrations of 91% of nominal.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in daphnia test medium. The Limit of Quantification was 1.33 mg thymol/L in daphnia test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in daphnia test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in daphnia test medium.

KCP 5.1.2/11 (A 2.3.1/19 of this dRR)

This study has been submitted within the first approval dossier of active substances (RMS: UK) and/or for registration of product in SEU (see part B0 for Regulatory history of active substances and product).

Comments of zRMS:	The validation of the method for analysis of thymol in alga test medium was previously evaluated at EU level.
-------------------	---

Data point:	CP 5.1.2/11 (A 2.3.1/19 of this dRR)
Report author	Grade R., Wydra V.
Report year	2008c
Report title	Toxicity of 3AEY to <i>Pseudokirchneriella subcapitata</i> in an Algal Growth Inhibition Test
Report No	34303210
Document No	Not applicable
Guidelines followed in study	Commission Directive 92/69/EC, Part C, C.3 OECD 201 EPA OPPTS 850.5400
Deviations from current test	No information on the daughter ions monitored is presented in the

guideline	report. However, the test is performed with analytical grade reference item and therefore identity of the analyte is confirmed. Validation is in agreement with Guidance Document SANCO/3029/99 rev.4.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) under data point IIIA 10.2.2.3/01
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

Although the study report refers to 3AEY which contains eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Test item

Name:	3AEY
Source and lot/batch no.:	Eden Research plc, lot n°YPOA3Y424-1.1
Active substance content:	Thymol (CAS no. 89-83-8): 6.47% w/w (64.7 g/kg) Geraniol (CAS no. 106-24-1): 6.56% w/w (65.6 g/kg) Eugenol (CAS no. 97-53-0): 3.23 % w/w (32.3 g/kg)
Expiry date of lot/batch:	December 2008
Storage conditions:	Ambient temperature

Test Standards

Name:	Thymol crystals
CAS number:	89-83-8
Source and lot/batch no.:	Sigma-Aldrich, lot n°94747
Active substance content:	99.7% (equivalent to 997 g/kg)
Expiry date of lot/batch:	March 2008
Storage conditions:	Ambient temperature, dark

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6890 Series Gas Chromatograph with mass spectrometry detector
Analytical column:	HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness
Oven:	60°C (2 min) – 10°C/min – 100°C (10 min) – 20°C/min – 240°C (0 min)
Injection volume:	1 µL
Injection mode:	Splitless mode
Carrier gas:	Helium 5.0
Flow rate:	Not stated
Retention time:	Thymol: Approx. 15 min
Monitored ions/transitions:	Not stated

Sample preparation

Samples of test water are diluted with acetonitrile if necessary, to fit within the calibration range prior to quantification. Test item concentration was 1.0, 3.2, 10.0, 32.0 and 100 mg/L. With respect to thymol, this is equivalent to 0.065, 0.21, 0.65, 2.07 and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w.

Stock solutions and calibration standards

Thymol reference item was used to prepare a 1 g/L stock in acetonitrile. This stock was further diluted in

acetonitrile to provide a combined secondary standards stock of concentration 50 mg/L containing all three substances (eugenol, geraniol and thymol), although only thymol is considered in this summary. From this secondary stock, calibration standards spanning the concentration range 0.01 to 5 mg thymol/L are prepared in acetonitrile/test water (50/50, v/v).

Recovery and precision samples

About 50.0 mg of the test item were dissolved in 50 mL pure water to obtain a stock solution of 1 g test item/L. Appropriate amounts of this stock solution were diluted with test water to obtain fortified samples at a level of 1, 10 and 100 mg test item/L. With respect to thymol, this is equivalent to 0.065, 0.65, and 6.47 mg/L respectively, based on a thymol content of 6.47% w/w.

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation : % of nominal = $(c/c_{\text{nom}}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Linearity:

Linearity was investigated over the range 0.01 – 5 mg/L through two separate concentration ranges: Low level range = 0.01 – 0.5 mg/L and high-level range = 0.5 – 5 mg/L.

Six calibration standards were quantified. The equation to the calibration line was $507512x - 9179$, the correlation coefficient R was 0.9963 (y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is considered validated for this range of concentration.

Specificity:

Specificity was assessed through analysis of blank test medium and solvent blanks, as well as certified reference material. No interferences > 30% were observed. The method is specific.

Copies of relevant chromatograms are provided for thymol calibration standards, control solutions, fortification solutions and test solutions.

LOD, LOQ:

The Limit of Detection (LOD) of the method is defined as the lowest concentration having a peak height equivalent to or better than three times the baseline noise. The LOD was found at 0.005 mg/L.

The Limit of Quantification (LOQ) was determined as the lowest fortification level at which acceptable mean recovery (70 to 110% of nominal) with a relative standard deviation (RSD) < 20% was obtained. The LOQ was found at 1 mg test item/L, corresponding to 0.065 mg thymol/L.

Accuracy, Repeatability (precision):

Accuracy was verified at four levels: 1.0, 10.0 and 100 mg/L, corresponding to 0.065, 0.65 and 6.47 mg thymol/L respectively.

Test item fortification level (mg/L)	Accuracy	%RSD	n
1	109	11.7	4
10	100	3.2	4
100	90	4.3	4

Overall	101	9.2	12
---------	-----	-----	----

The data indicates that the accuracy and precision of the method is acceptable at a 1.0 mg test item/L and above in that the mean recoveries are well within the range (70 - 110%) and the RSD obtained is well below the limit value of 20%. The LOQ is therefore set at 1.0 mg test item/L or 0.065 mg thymol/L.

Stability:

Samples were analysed directly after sampling; if that was not possible, samples were kept at < -10°C in the dark until analysis. Stability was not formally investigated.

For the determination of the stability of the test item under the test conditions and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the controls (containing algae) were taken at the end of the test (after the 96 hours test period). After 96 hours of exposure the mean measured test item concentrations were 89% (78 - 114%) of the nominal values. Thus, during the test period of 96 hours the algae were exposed to mean measured concentrations of 88% of nominal. All reported results in the report amendment are expressed in terms of the geometric mean concentrations of the test item.

Conclusions

Sample of aqueous test medium are either injected as is or diluted in acetonitrile to fit within the calibration range prior to analysis of thymol content by GC-MS.

The method described was acceptably validated at concentration levels relevant to the test results. The method is linear, accurate and precise and suitable for the quantification of thymol in alga test medium. The Limit of Quantification was 0.065 mg thymol/L in alga test medium.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in alga test medium was previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. the method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in alga test medium.

KCP 5.1.2/12 (A 2.3.1/20 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of thymol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level.</p> <p>The HPLC-UV/DAD analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW was fully validated, according to SANCO/3029/99 rev.4 guidance document.</p> <p>The Limit of Quantification of thymol was 41.6 mg/kg in sugar feeding solution and 199.9 mg/L in water stock solution.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with ≤ 20% RSD).</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.1.2/12 (A 2.3.1/20 of this dRR)
Report author	Aversa S
Report year	2019
Report title	Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document)
Report No	BT081/19

Document No	Not applicable
Guidelines followed in study	SANCO 3029/99/rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Water stock solution

Sample of aqueous stock solution are diluted in acetonitrile to fit within the calibration range prior to analysis by HPLC-DAD.

50% w/v sugar feeding solution

The 50 % w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test item

Name:	ARAW
Source and lot/batch no.:	Eden Research plcplc, lot n°BT-89
Active substance content:	Eugenol: 3.29% w/v Geraniol: 6.46% w/v Thymol: 6.57% w/v
Product density:	1.027 kg/L
Expiry date of lot/batch:	October 2020
Storage conditions:	Ambient, dark.

Analytical standard

Name:	Thymol
Source and lot/batch no.:	Sigma Aldrich, lot n°STBH8227
Active substance content:	>99.9%
Expiry date of lot/batch:	September 2021
Storage conditions:	Ambient, dark.

Origin of samples

The method was used to analyse the water stock solution coming from the ecotoxicological study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure, Aversa S, 2019) and the sugar feeding solution coming from the ecotoxicological study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees *Apis mellifera* L., 10-day feeding laboratory test, Pecorari F., 2019).

Analysis parameters

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile

Gradient: Isocratic 60% A/ 40% B
Flow rate: 0.6 mL/min
Retention time: Approx. 2.7 min
Detector wavelength: 210 nm

Sample preparation

Water stock solution

The water stock solutions originate from study BT060/19 (Effects of ARAW to honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure, Aversa S, 2019).

The nominal concentration of the test water stock solutions is 100 g product/L at high level and 6.25 g product/L at low level.

The high-level stock solution is diluted with acetonitrile, first 0.4 mL in 10 mL to produce a 4 g product/L solution, then 1 mL in 10 mL to produce a final sample of concentration 0.4 g product/L.

The low-level stock solution is diluted with acetonitrile 0.6 mL in 10 mL to produce a final sample of concentration 0.375 g product/L. Thymol concentration in the diluted stocks is presented below:

ARAW content in dilution (g/L)	Thymol content in ARAW	Thymol content in dilution (mg)	Thymol extract concentration (mg/L)
0.4	6.57% w/v or 63.97 mg/g (density 1.027)	25.59	25.59
0.375		23.99	23.99

Sugar feeding solution

The test sugar feeding solution originates from study BT059/19 (Chronic oral effects of ARAW on adult worker honeybees *Apis mellifera* L., 10-day feeding laboratory test, Pecorari F., 2019).

The nominal concentration of the sugar feeding solution is 50000 mg product/kg (equivalent to 50 mg product/g - high level) and 1300 mg product/kg (equivalent to 1.3 mg product/g - low level).

Samples of test feeding solution (1 g) are mixed with water (2 mL) and acetonitrile (10 mL) and sonicated. Phase separation is obtained by the addition of sodium chloride, shaking and settling. The acetonitrile phase is collected and filtered. At that point, extract concentration is 0.13 mg product/mL low level and 5 mg product/mL high level. The low-level extract is analysed directly, while the high-level extract is further diluted (1 mL in 10 mL) to produce an extract concentration of 0.5 mg product/mL prior to analysis by HPLC-DAD.

High- and low-level extract thymol concentrations are presented below:

ARAW content in extract (g/L)	Thymol content in ARAW	Thymol content in dilution (mg)	Thymol extract concentration (mg/L)
0.13	6.57% w/v or 63.97 mg/g (density 1.027)	8.316	8.316
0.50		31.98	31.98

Blank feeding solutions are subjected to the same procedure to produce a blank matrix extract used for dilution of standards for matrix effect investigation.

Stock solutions and calibration standards

100.4 mg of thymol analytical standard were weighted in a 10 mL volumetric flask, dissolved and made up to volume with methanol. This Stock Solution was named SS1Thy and had a thymol concentration of 10029.96 mg/L.

2 mL of this stock was transferred to a 10 mL volumetric flask and diluted to volume with acetonitrile to produce a 2005.992 mg/L stock (identification: SS2 mix).

Linearity standards – water stock solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (Thymol 200.5992 mg/L). From this stock, five linearity standards spanning the range 3 – 70 mg/L were prepared in acetonitrile.

Linearity standards – sugar feeding solution:

In a 10 mL volumetric flask, 1.0 mL of SS2 mix was transferred and diluted to 10 mL with acetonitrile to have the SS3 mix solution (Thymol 200.5992 mg/L). From this stock, five linearity standards spanning

the range 1 – 70 mg/L were prepared in acetonitrile.

Recovery and precision samples

Water stock solution

Recovery and precision samples are prepared at two levels in water: 120 000 mg product /L and 3125 mg product /L. These solutions were diluted in acetonitrile to obtain the following concentrations:

High-level accuracy: 1 mL in 10 mL followed by 0.25 mL in 10 mL to afford a final concentration of 0.3 g product/L.

Low-level accuracy: 1 mL in 10 mL to afford a final concentration of 0.313 g product/L.

High- and low-level accuracy samples thymol concentrations are presented below:

ARAW content in extract (g/L)	Thymol content in ARAW	Thymol content in dilution (mg)	Thymol extract concentration (mg/L)
0.3	6.57% w/v or 63.97 mg/g (density 1.027)	19.19	19.19
0.3125		19.99	19.99

Sugar feeding solution

A 6329.19 mg product/L stock solution is prepared in water to use as fortification solution for the sugar feeding solution.

Blank sugar feeding solution is fortified levels using the aforementioned stock solution in water to produce fortified samples at 60000 mg product/kg (equivalent to 60 mg product/g - high level) and 650 mg product/kg (equivalent to 0.65 mg product/g - low level). Fortified sugar feeding solutions are extracted as described above to produce extract of concentrations 0.065 mg product/L (low-level) and 6 mg product/L (high level). The low-level extract is analysed directly while the high-level is further dilute 1 mL in 10 mL with acetonitrile.

High- and low-level accuracy samples thymol concentrations are presented below:

ARAW content in extract (g/L)	Thymol content in ARAW	Thymol content in dilution (mg)	Thymol extract concentration (mg/L)
0.065	6.57% w/v or 63.97 mg/g (density 1.027)	4.16	4.16
0.6		38.38	38.38

Calculations

Samples were quantified by measuring the peak area with reference to the calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentrations in mg/L. The correlation was performed using a linear regression function of the type $y = ax + b$

Where:

y = peak area

x = concentration of the test item in the samples [mg/L]

a= slope

b = y-axis intercept

The concentration of the test item in the samples was calculated using the equation $c = x \times d$

Where:

c = test item concentration in samples [mg/L]

x = concentration found in injected samples [mg/L]

d = dilution factor

The recovery rate was calculated using the equation: % of nominal = $(c/c_{nom}) \times 100\%$

Where:

c = measured concentration of the test item in the sample [mg/L]

c_{nom} = nominal concentration of the test item [mg/L]

Findings

Matrix effects:

Matrix effect were investigated by preparing two calibration standards either in matrix (water or sugar feeding solution extract) or in acetonitrile and comparing response for suppression or enhancement. No

significant (>20%) suppression nor enhancement was observed in any of the matrices and calibration standards were prepared in acetonitrile.

Specificity:

The specificity for the three analytes was established by the comparison of the UV Spectrum of the standard solution and a high level recovery solution. In addition, specificity was assessed through analysis of blank test medium and solvent blanks. No interferences > 30% were observed. The method is specific.

	Water stock solution		Sugar feeding solution	
Linearity	3 – 70 mg/L, n = 5 Y = 10.4300x – 1.5277 R = 0.9999		1 – 70 mg/L, n = 5 Y = 10.4622x – 1.7210 R = 1.0000	
Accuracy	120000 mg prod./L Eq. to 7676 mg thymol/L	3125 mg prod./L Eq. to 199.9 mg thymol/L	60000 mg prod/kg Eq. to 3838 mg thymol/L	650 mg prod./kg Eq. to 41.58 mg thymol/kg
	97.43 %	96.03 %	99.64 %	96.42 %
%RSD	1.45 %	3.61 %	1.89 %	2.61 %
LOQ	3125 mg prod./L Eq. to 199.9 mg thymol/L		650 mg prod./kg Eq. to 41.58 mg thymol/kg	
LOD	3 mg/L (lowest calibration level)		1 mg/L (lowest calibration level)	

The method is accurate and precise at a level of 41.6 mg/L and above in sugar feeding solution and at 199.9 mg/L in water stock solution.

Stability:

Stability was not investigated.

Conclusions

Thymol is extracted from test medium (sugar feeding solution and water stock solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of thymol content is linear, accurate and precise and suitable for the quantification of thymol in aqueous stock solution and sugar feeding solution. The Limit of Quantification was 41.6 mg/kg in sugar feeding solution and 199.9 mg/L in water stock solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in aqueous stock solution and sugar feeding solution was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in aqueous stock solution and sugar feeding solution.

KCP 5.1.2/14 (A 2.3.1/21 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of thymol in aqueous stock solutions from bee larvae chronic studies was not previously evaluated at EU level.</p> <p>The method for quantification of thymol in aqueous stock solution used in the 22-day larval toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19).</p> <p>The LOQ of thymol was 41.58 mg/kg in water stock solution.</p> <p>The study is acceptable.</p>
-------------------	---

Method type	HPLC-DAD
Instrument:	Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column:	Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature:	30°C
Injection volume:	2 µL
Eluent A:	Water with 0.1% trifluoroacetic acid
Eluant B:	Acetonitrile
Gradient:	Isocratic 60% A/ 40% B
Flow rate:	0.6 mL/min
Retention time:	Thymol: Approx. 2.7 min

Detector wavelength: 210 nm

Method validation

The method for quantification of thymol in aqueous stock solution used in the 10-day chronic adult toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Thymol is extracted from test medium (sugar feeding solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of thymol content is linear, accurate and precise and suitable for the quantification of thymol in sugar feeding solution. The Limit of Quantification was 41.58 mg/kg in sugar feeding solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in sugar feeding solutions.

Sugar feeding solution (Ecotoxicology)

KCP 5.1.2/13 (A 2.3.1/22 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of thymol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level.</p> <p>The concentrations of the active substances eugenol, geraniol and thymol in feeding solutions were analyzed.</p> <p>The analysis of samples was performed following the analytical method validated in a dedicated GLP study BT081/19, in compliance with the guideline SANCO/3029/99 rev. 4.</p> <p>The LOQ of thymol was 41.58 mg/kg in sugar feeding solution.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/13 (A 2.3.1/22 of this dRR)
Report author	Pecorari F.
Report year	2019a
Report title	Chronic oral effects of ARAW on adult worker honeybees <i>Apis mellifera</i> L., 10-day feeding laboratory test
Report No	BT059/19
Document No	Not applicable
Guidelines followed in study	OECD Guideline for the testing on chemicals 245 "Honey bee (<i>Apis mellifera</i> L.), Chronic Oral Toxicity test (10-day feeding test in the laboratory)".
Deviations from current test guideline	None.
Previous evaluation	No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

The 50% w/v sugar feeding solution containing 0.2% xanthan gum is mixed with water and acetonitrile, then acetonitrile is phase-separated by the addition of salts, filtered, diluted if necessary and quantified by HPLC-DAD.

Although the study report refers to ARAW (alternative name of 3AEY) which contains eugenol, geraniol and thymol, only thymol is considered in this summary.

Materials and methods

Test material

Name: ARAW
Source and lot/batch no.: Eden Research plc, lot n°BT-89
Active substance content: Eugenol (CAS no. 97-53-0): 3.29% w/v (32.9 g/L)
Geraniol (CAS no. 106-24-1): 6.46% w/v (64.6 g/L)
Thymol (CAS no. 89-83-8): 6.57% w/v (65.7 g/L)
Product density: 1.027 kg/L
Expiry date of lot/batch: October 2020
Storage conditions: Ambient, dark.

Analytical standard

Name: Thymol
Source and lot/batch no.: Sigma Aldrich, lot n°STBH8227
Active substance content: > 99.9%
Expiry date of lot/batch: September 2021
Storage conditions: Ambient, dark.

Analysis parameters

Method type: HPLC-DAD
Instrument: Agilent HPLC with DAD detector 1200 series with Chemstation and software OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08
Analytical column: Agilent Poroshell 2.7 µm SB-C18 3.0 x 50 mm
Column temperature: 30°C
Injection volume: 2 µL
Eluent A: Water with 0.1% trifluoroacetic acid
Eluant B: Acetonitrile
Gradient: Isocratic 60% A/ 40% B
Flow rate: 0.6 mL/min
Retention time: Thymol: Approx. 2.7 min
Detector wavelength: 210 nm

Method validation

The method for quantification of thymol in aqueous stock solution used in the 10-day chronic adult toxicity test was validated separately in Aversa 2019 (Validation of an analytical method for the determination of eugenol, geraniol and thymol in water stock solution and in sugar feeding solution treated with test item ARAW (according to SANCO 3029/99/rev.4 guidance document), report number BT081/19), which is presented in this submission.

Conclusions

Thymol is extracted from test medium (sugar feeding solution) with acetonitrile prior to analysis by HPLC-DAD.

The method described was acceptably validated at concentration levels relevant to the test results. The method for the determination of thymol content is linear, accurate and precise and suitable for the

quantification of thymol in sugar feeding solution. The Limit of Quantification was 41.58 mg/kg in sugar feeding solution.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in sugar feeding solutions from adult bees chronic toxicity study was not previously evaluated at EU level. It was performed under GLP and followed requirements of SANCO/3029/99 rev.4. The method was successfully validated at concentration levels relevant to the test's results.

The method is acceptable for the quantification of thymol in sugar feeding solutions.

Water, buffer solutions,... (Properties)

KCA 4.1.2/25 (A 2.3.1/23 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis of thymol in aqueous and n-octanol solutions was not evaluated at EU level.</p> <p>The n-octanol/water partition coefficient of the test item was determined according to OECD guideline 107 and EC method A.8.</p> <p>The test was performed at 3 different ration of n-octanol and water buffered at 3 different pH values (pH 4, 7 and 9). The determined value of the log of the partition coefficient was within the acceptable range of ± 0.3 log units.</p> <p>The contents of thymol in aqueous and n-octanol solutions were determined by HPLC with UV detection.</p> <p>The analytical method was validated following SANCO/3029/99, rev.4 with regard to linearity of detector response, precision, accuracy and non-analyte interference of the analytical system.</p> <p>Mean recoveries and relative standard deviations were in the range 70-110% with $\leq 20\%$ RSD).</p> <p>The limit of quantification (LOQ) for thymol in aqueous phase was 0.1265 mg/L.</p> <p>The limit of quantification (LOQ) for thymol in n-octanol phase was 3.380 mg/L.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CA 4.1.2/25 (A 2.3.1/23 of this dRR)
Report author	Lingott J
Report year	2020
Report title	Partition coefficient of Thymol (Shake-Flask Method)
Report No	S20-06644
Document No	Not applicable
Guidelines followed in study	EEC A8, OECD 107
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

The contents of thymol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

Test material

Analytical grade of thymol was used as test item.

Test material

Name: Thymol, technical grade
CAS number: 89-83-8
Source and lot/batch no.: Dr. Ehrenstorfer, lot G161924,
Substance content: 99.69% (equivalent to 996.9 g/kg)
Expiry date of lot/batch: 05.12.2023
Storage conditions: Ambient, dark.

Analysis parameters

Method type: HPLC-UV
Instrument: Agilent 1260 Infinity
Analytical column: Cadenza 5CD-C18, 150 x 4.6 mm, 5 µm, Imtakt
Column temperature: 40°C
Detector wavelength: 277 nm, bandwidth: 4 nm
Injection volume: 20 µL
Eluent A: Acetonitrile
Eluent B: Ultra-pure water containing 0.1% phosphoric acid
Flow rate: 1 mL/min

Gradient

Time [min]	% A	% B
0.0	65	35
5.0	65	35
6.0	90	10
8.0	90	10
9.0	65	35
13.0	65	35

Retention time: Thymol: Approx. 4.3 min

Sample preparation

pH 4, pH 7 and pH 9 buffers are prepared and saturated in n-octanol prior to the test. Correspondingly, samples of n-octanol are saturated with individual buffer solutions.

Stock solutions of concentration 1000 mg/L are individually prepared in buffer-saturated octanol. These stocks are combined with the corresponding octanol-saturated pH 4, pH 7 or pH 9 buffer and equilibrated as part of the test. The concentration of the test item was determined in both phases. To minimize the risk of including traces of n-octanol into the aqueous phases, the n-octanol phase was removed completely before sampling the aqueous phase.

Prior to analysis, the aqueous phase was diluted by a factor of two with acetonitrile, while the octanol phase was diluted by a factor of 200 with acetonitrile. Diluted samples were quantified by HPLC-UV.

Calibration standards preparation

A stock solution containing 1 mg/mL was prepared in acetonitrile. This stock was sequentially diluted in acetonitrile to produce analytical standards within the range 0.1 – 10 mg/L.

Procedural recovery samples

Procedural recovery samples were prepared either in n-octanol or in a specifically prepared buffer mix.

Aqueous phase, low recovery

0.5 mL of a low spike solution containing 0.251 mg/L thymol in acetonitrile were transferred into a 1.5 mL vial and 0.5 mL buffer mix were added.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of thymol in the aqueous phase is 0.1255 mg/L.

Aqueous phase, high recovery

0.5 mL of a high spike solution containing 0.603 mg/L thymol in acetonitrile were transferred into a 1.5 mL vial and 0.5 mL buffer mix were added.

This corresponds to a final dilution factor of 2. The sample was directly used for analysis. Five samples were prepared. The nominal content of thymol in the aqueous phase is 0.3015 mg/L.

n-Octanol phase, low recovery

To 2 mL of a low spike solution containing 676.0 mg/L thymol in acetonitrile 2 mL n-octanol were added. The samples were filled up to a volume of 20 mL with acetonitrile. In a second dilution step 1 mL of this solution was filled up to a final volume of 20 mL with acetonitrile.

This corresponds to a final dilution factor of 200. The sample was directly used for analysis. Five samples were prepared. The nominal content of thymol in the n-octanol phase is 3.380 mg/L.

n-Octanol phase, high recovery

2 mL of a high spike solution containing 1352 mg/L thymol in acetonitrile were transferred into a vial and 2 mL n-octanol were added. The samples were filled up to a volume of 20 mL with acetonitrile. In a second dilution step 1 mL of this solution was filled up to a final volume of 20 mL with acetonitrile.

This corresponds to a final dilution factor of 200. The sample was directly used for analysis. Five samples were prepared. The nominal content of thymol in the n-octanol phase to 6.760 mg/L.

Calculations

The concentration of analyte in the measured sample was calculated by the following equations:

$$C = \left(\frac{(A - b)}{a} \right) \cdot d \cdot \frac{v_{std}}{v_s}$$

Where:

$A = a \cdot C + b$ (calibration equation)

A = response ['Area']

b = Y-axis intercept of the calibration curve ['Area']

a = slope of the calibration curve ['Area'/(mg/L)]

C = concentration of analyte in measuring sample [mg/L]

d = dilution factor

v_{std} = injection volume of standards

v_s = injection volume of sample

The amount of analyte in the measuring sample was calculated by the following equations:

$$M = \frac{C \cdot V}{1000 \text{ mL/L}}$$

Where:

M = amount of analyte in measuring sample [mg]

V = volume of the aqueous or n-octanol phase [mL]

The calibration curve was determined from standards of analyte which were measured parallel to the samples.

Findings

Linearity:

The method was found to be linear over the range 0.1 – 10 mg/L for thymol at each pH. Six calibration standards were quantified. The equations to the calibration line were respectively:

- at pH 4: $1.613979x - 0.01115$ and the correlation coefficient $R = 1.0000$,
- at pH 7: $1.606890x - 0.000798$ and the correlation coefficient $R = 1.0000$,
- at pH 9: $1.614995x - 0.001529$ and the correlation coefficient $R = 1.0000$,

(y = peak area of thymol, x = concentration of thymol (in mg/L)).

The linearity of the method is validated for this range of concentration at different pH.

Specificity:

Blank samples of the buffers and n-octanol were prepared as described above and quantified by HPLC-UV. No signal was observed in any of the blank samples at the retention time of thymol. No interferences were observed.

Copies of relevant chromatograms are provided for standards of thymol, blank samples containing buffer mix, blank samples containing n-octanol, aqueous phase of sample with buffer pH 4, 7 and 9, n-octanol phase of sample with buffer pH 4, 7 and 9, and recovery samples for n-octanol phase and for aqueous phase.

LOD, LOQ:

The limit of quantification (LOQ) for thymol in aqueous phase after dilution was 0.12565 mg/L (lowest

recovery concentration), corresponding 0.251 mg/L in the initial aqueous phase sample.
The limit of quantification (LOQ) for thymol in n-octanol phase after dilution was 3.380 mg/L (lowest recovery concentration), corresponding 676.0 mg/L in the initial aqueous phase sample.
The limit of detection (LOD) thymol was 0.1 mg/L (nominal concentration of the lowest calibration standard).

Accuracy, Repeatability (precision):

Phase	Recovery level (mg/L)	Recovery (%)	%RSD	Horrat value	n
Aqueous	0.251	100.2	1.1	0.08	5
	0.603	101.4	0.2	0.02	5
Overall		100.8	1.0	-	10
n-Octanol	676	99.5	0.3	0.07	5
	1352	99.2	0.7	0.19	5
Overall		99.3	0.5	-	10

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries are well within the range (80 -120%) and the Horrat values obtained are well below the limit value of 1.

Conclusions

The contents of Thymol in aqueous and n-octanol solutions were determined by HPLC with UV detection.

The method for quantification of thymol in solutions from water/n-octanol partition coefficient testing was fully validated for specificity, linearity, accuracy and precision. The method is acceptable for the quantification of thymol in water/n-octanol partition coefficient testing solutions.

Assessment and conclusion by applicant:

The validation of the method for analysis of thymol in aqueous and n-octanol solutions was performed under GLP according to Guideline SANCO/3030/99 rev.4 and was successfully validated. Validation also complies with SANCO/3030/99 rev.5.

The method is acceptable for the quantification of thymol in physicochemical testing solutions.

A 2.3.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.3.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

GRAPES

KCP 5.1.2/05 (A 2.3.2.1/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method was not previously evaluated at EU level.</p> <p>Grape bunches samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANCO/3029/99, rev.4 for grapes matrix in the EAS Study S20-06528.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in grapes is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations, except for geraniol and thymol where the blank value was around 50%.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The study is acceptable.</p>
-------------------	--

Data point:	CP 5.1.2/05 (A 2.3.2.1/01 of this dRR)
Report author	Chadwick G
Report year	2021a
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to grapevine, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F, 2021, report n°S20-06337-L1]
Report No	S20-06337
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified after extraction from grape samples, by GC-MS (A single ion is monitored however a complete method validation study including three thymol ions is currently ongoing).
The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.
Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical standard

Name:	Thymol
CAS No.:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, lot n°STBJ4851
Active substance content:	99.9%
Expiry date of lot/batch:	October 2022
Storage conditions:	Ambient, dark.

Analysis parameters

Method type	GC-MS
Instrument:	Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column:	Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven:	100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature:	280°C
Injection volume:	1 µL
Injector temperature:	250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas:	Helium
Flow rate:	1.1 mL/min
Ionisation mode	Electron Impact Ionisation (EI)
Acquisition type:	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Retention time:	Thymol: approx. 16 min

Monitored ions (m/z)

Thymol: 91 (30 ms dwell) for quantification.

No confirmation ion was monitored, as the specificity of the method was confirmed as part of method validation report N° S20-06528 [Driss F., 2021] included in this submission.

Sample preparation

Extraction: An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step.

For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO_4 , 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 μL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of thymol: the thymol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 $\mu\text{g/mL}$.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 $\mu\text{g/mL}$ in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract or solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of grape were fortified prior to extraction with the fortification solutions as described below. Two fortification solutions of concentrations 10.0 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$ were prepared from the calibration stock solution and these solutions were used respectively to spike control grape samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) \times A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If needed A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak area of control sample = $A_A - (\text{mean})$ blank peak area
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the thymol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 150) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of thymol residues in grape and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $405x + 5764$, the correlation coefficient R was 0.9955 (y = peak area of thymol, x = concentration of thymol (in ng/mL)).

The linearity of the method is validated for this range of concentration of thymol.

Specificity:

Specificity was studied by analysis samples of standards of thymol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with thymol, treated grape sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of thymol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of thymol, matrix-matched standards solutions for grape and grape bunches, and control (untreated) grape sample, control grape sample spiked with thymol, treated grape sample for the quantification ions (m/z 150).

In addition, a second method involving a different column was used to confirm analyte identification.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in grape.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions of the grapes for the quantification ions (m/z 150).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	92	20	5
0.10	98	3	6
Overall	95	13	11

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 - 120%) and the RSD obtained is well below the limit value of 20%.

Conclusions

Thymol is quantified after extraction from grape samples, by GC-MS (One ion monitored).

This analytical method for the determination of thymol content in grapes samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in grapes.

The method presented herewith is satisfactory and can be applied to quantify thymol in grapes.

The method used was fully validated in the Eurofins study S20-06528 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated. The method is acceptable for the quantification of thymol in grapes.

KCP 5.2/01 (A 2.3.2.1/02 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in grapes was not previously evaluated at EU level.</p> <p>The analytical method has been fully validated for the determination of eugenol, methyl-eugenol, thymol and geraniol in grapes samples according to the SANTE/2020/12830, rev.1. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification was 0.01 mg/kg for eugenol, methyl-eugenol, thymol and geraniol in grapes.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation below 20%.</p> <p>The studies are acceptable.</p>
-------------------	--

Data point:	CP 5.2/01 (A 2.3.2.1/02 of this dRR)
Report author	Driss F
Report year	2021a
Report title	Validation of Residue Method for the Determination of Eugenol,

Report No	Geraniol, Thymol and Methyl-Eugenol in Grape S20-06528
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830, rev.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS and LC-MS/MS.

The method was used in Eurofins studies S20-06337 and S20-06526 in the same test facility (Eurofins Agroscience Services Chem SAS, France), which are included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS																
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)																
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)																
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)																
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz																
Purge Flow	50 mL/min at 0.05 min																
Injection volume	1 µL (depending on sensitivity)																
Injector temperature	250°C																
Column oven temperature programme	<table><tr><th>Step</th><th>Rate [°C/min]</th><th>Temperature [°C]</th><th>Hold time [min]</th></tr><tr><td>1</td><td>-</td><td>100</td><td>2.0</td></tr><tr><td>2</td><td>5.0</td><td>160</td><td>0.0</td></tr><tr><td>3</td><td>15</td><td>250</td><td>0.0</td></tr></table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0	2	5.0	160	0.0	3	15	250	0.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]														
1	-	100	2.0														
2	5.0	160	0.0														
3	15	250	0.0														
Retention time:	Approx. 16.0 min for thymol																
Ionisation mode	Electron Impact Ionisation (EI)																
Scan type	SIM (Selected Ion Monitoring)																
Ion source temperature	230°C																
Quadrupole temperature	150°C																

Solvent delay 6 min
Ions monitored Thymol: 91 (100 ms dwell)

Chromatographic conditions for confirmation of Thymol

Method type LC-MS/MS
HPLC system LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar)
LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure 200 bar
Autosampler temperature 4°C
Column oven temperature 60°C
Injection volume 10 µL (depending on sensitivity)
Mobile phases Eluent A: Acetonitrile;
Eluent B: Water containing 5mM of ammonium acetate
Gradient

Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
0.0	25	75	0.6
0.5	25	75	0.6
3.0	95	5.0	0.6
4.5	95	5.0	0.6
5.5	25	75	0.6
7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste
Retention time(s) Approx. 3.02 min for thymol

Mass spectrometric conditions

MS system SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)
SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation type Electrospray ionisation (ESI, TurboIonSpray)
Polarity Negative ion mode
Scan type MS/MS, Multiple Reaction Monitoring (MRM)
Capillary voltage (IS) -4500 V
Ionspray turbo heater 100 °C
(TEM)
Curtain gas (CUR) Nitrogen set at [25-40] psi Gas flow 1 (GS1) Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)
Collision gas (CAD) Nitrogen set at 8 psi Gas flow 2 (GS2) Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)
Analyte monitored Mass transition monitored (*m/z*) Declustering potential (DP) [V] Entrance potential (EP) [V] Collision energy (CE) [eV] Cell exit potential (CXP) [V] Dwell time [ms]
Thymol 149 → 134 (neg) -85 -10 -20 -15 35

Sample preparation

An amount of 10 g \pm 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μ L of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.1 μ g/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.0 μ g/mL.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 μ g/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of grapes which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 μ g/mL (equivalent to 0.0025 to 0.50 mg/kg of thymol in initial grapes samples).

All solutions were stored at 1 °C to 10 °C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of grapes on the GC-MS and LC-MS/MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
$C_{\text{Solv-Std}}$	Nominal concentration of standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 70 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was

evaluated.

Extract stability

Extracts were quantified and stored for 7 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = $A_A - (\text{mean})$ blank peak area
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by using two separate analytical methods and monitoring separate fragment ions/mass transitions: GC-MS with fragment ion m/z 91 and LC-MS/MS with mass transition 149 → 134.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Thymol (%)	
		Quantification (m/z 91)	Confirmation (m/z 149→134)
Grapes	80	(+) 4.6	(-) 26.4

For the confirmatory method, matrix suppression or enhancement was $\geq 20\%$ in Grapes for thymol thus deemed to be significant.

Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$ ($n = 8$), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for each method:

- m/z 91, GC-MS: $y = 229x + 297$; $r = 0.9977$, $r^2 = 0.9954$
- mass transition 149 \rightarrow 134, LC-MS/MS: $151x - 1190$; $r = 0.9984$, $r = 0.9968$

Both methods are linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for both methods.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.002 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in grape matrix.

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 91 (Proposed for Quantification)*							
Grapes	0.01	109; 123; 113; 99; 107	110	8	5	110	7
	0.1	110; 123; 106; 105; 108	110	7	5		
Transition m/z 149→134 (Proposed for Confirmation)							
Grapes	0.01	99; 93; 98; 95; 100	97	3	5	95	5
	0.1	95; 100; 91; 87; 92	93	5	5		

*Observable peak was detected in control sample extract (interference around 50% of LOQ). Recoveries are corrected for the mean peak areas of the control sample extracts

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of thymol, control matrix, and control matrix spiked with thymol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the thymol.

For the fragment ion, a blank correction was performed with the mean peak area of the control sample(s) used for fortification for the ion monitored for thymol (interference around 50% of LOQ).

A blank correction was performed with the mean peak area of the control sample(s) used for fortification for the ion monitored in GC-MS for thymol (interference around 50% of LOQ). Recoveries and matrix-matched standards were corrected by background subtraction. In addition, one ion was monitored and quantified by GC-MS and one mass transition by LC-MS/MS. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for the ion and transition monitored. Moreover, mass spectra of thymol are provided to justify the choice of ion and ion transition monitored.

Stability of standards and extracts:

Stock stability: The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1 °C to 10 °C for 70 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts: Thymol was found to be stable in final extracts of grape for 7 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Thymol - Fragment m/z 91 (Proposed for Quantification)**						
Grape	0	0.01	109; 123; 113; 99; 107	110	8	(-) 18
	7		73; 97, 90, 77, 112	90	17	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

**Observable peak was detected in control sample extract (interference around 50% of LOQ). Recoveries are corrected for the mean peak areas of the control sample extracts

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Thymol is extracted from grape matrix using acetonitrile followed by solid-phase extraction clean-up, then quantified by GC-MS or LC-MS/MS using one fragment ion for quantification and one mass transition for confirmation of method specificity.

This analytical method for the determination of thymol content in grape matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in grapes.

The method presented herewith is satisfactory and was applied to quantify thymol in grapes in Eurofins study S20-06337 and S20-06526 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in grapes was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1 and was successfully validated.

The method is acceptable for the quantification of thymol in grapes.

KCP 5.2/02 (A 2.3.2.1/03 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The study “<i>Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions</i>” was not previously evaluated at EU level.</p> <p>Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06528. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte.</p> <p>The study is acceptable for the quantification of thymol in grapes.</p>
-------------------	--

Data point:	CP 5.2/02 (A 2.3.2.1/03 of this dRR)
Report author	Driss F.
Report year	2021b
Report title	Storage Stability of Eugenol, Geraniol, Thymol and Methyl Eugenol in Grape under Deep Frozen Conditions
Report No	S20-06526
Document No	Not applicable

Guidelines followed in study SANTE/2020/12830 rev.1

Deviations from current test guideline None

Previous evaluation No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

Samples of grapes were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within Study n°S20-06528 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS																
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)																
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)																
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)																
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz																
Purge Flow	50 mL/min at 0.05 min																
Injection volume	1 µL (depending on sensitivity)																
Injector temperature	250°C																
Column oven temperature programme	<table border="1"> <thead> <tr> <th>Step</th> <th>Rate [°C/min]</th> <th>Temperature [°C]</th> <th>Hold time [min]</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>-</td> <td>100</td> <td>2.0</td> </tr> <tr> <td>2</td> <td>5.0</td> <td>160</td> <td>0.0</td> </tr> <tr> <td>3</td> <td>15</td> <td>250</td> <td>0.0</td> </tr> </tbody> </table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0	2	5.0	160	0.0	3	15	250	0.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]														
1	-	100	2.0														
2	5.0	160	0.0														
3	15	250	0.0														
Retention time:	Approx. 16.0 min for thymol																
Ionisation mode	Electron Impact Ionisation (EI)																
Scan type	SIM (Selected Ion Monitoring)																
Ion source temperature	230°C																
Quadrupole temperature	150°C																
Solvent delay	6 min																
Ions monitored	Thymol: 91 (30 ms dwell)																

Since the method was already validated for specificity, this study

only monitored a single ion.

Sample preparation

An amount of 10 g \pm 0.1 g of homogenised grape sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μ L of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified 0.1 mg/kg, eugenol concentration in the final extract was 1.0 μ g/mL.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 μ g/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 μ g/mL (equivalent to 0.0025 to 0.50 mg/kg of thymol in initial apples samples).

All solutions were stored at 1 °C to 10 °C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by single extraction and single injection.

Since the method had already been validated, matrix effects, LOQ was not investigated, and a single ion was monitored.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries ($R_{ProcRec}$) are calculated as follows:

$R_{Rel} (\%)$ and $R_{ProcRec} (\%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{Corrected}$) is calculated as follows:

$R_{Corrected} (%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0 analysis ($P_{Remaining}$) is calculated as follows:

$R_{Remaining} (%) =$	$\frac{R_{Rel_Mean}}{R_{Day0_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
R_{Day0_Mean}	Unrounded mean R_{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial grape samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Thymol	m/z 91: $y = 244x + 8744$; $r = 0.9989$, $r^2 = 0.9978$
--------	---

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.002 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0 storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The following recoveries were obtained:

Matrix	Fortification level (mg/kg)	Recovery (%)						Mean (%)	Rel. Std. Dev. (%)
		0 days	43 days	111 days	154 days	278 days	280 days		
		Thymol							
Grape	0.1	87, 98, 84	92	107	107	109	105 *	99	10

* Reanalysis performed to confirm results obtained at 278 days

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06528 for the quantification of residues of thymol in grape matrix was used to assess the stability of residues of thymol in grapes. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify thymol in grapes.

Assessment and conclusion by applicant:

The method used to verify the stability of thymol residue in grapes was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06528 and verified as part of this study. It was performed under GLP according to Guidelines SANTE/2020/12830 rev.1.

The method is acceptable for the quantification of thymol in grapes.

APPLES

KCP 5.1.2/06 (A 2.3.2.1/04 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol residues in apples was not previously evaluated at EU level.</p> <p>Apple fruit samples were analysed for residues of eugenol, methyl-eugenol, thymol and geraniol according to the analytical method that was previously validated according to SANCO/3029/99, rev.4 for apple matrix in the EAS Study S20-06529.</p> <p>The limit of quantification for eugenol, methyl-eugenol, thymol and geraniol in apple is set at 0.01 mg/kg.</p> <p>No residues above 30% of the LOQ were detected in the control (untreated) test portions used for recovery determinations.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.1.2/06 (A 2.3.2.1/04 of this dRR)
Report author	Chadwick G.
Report year	2021b
Report title	Determination of residues of eugenol, methyl eugenol, geraniol and thymol after 4 foliar applications of Mevalone (3AEY / EDN-004) to apple, 3 trials in N EU (3 x DEC) and 3 trials in S EU (3 x DEC), 2020 [Analytical phase by Driss F., 2021, report n° S20-06361-L1]
Report No	S20-06361
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

The method used was fully validated in the Eurofins study S20-06529 in the same test facility (Eurofins

Agroscience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical standard

Name: Thymol
CAS No.: 89-83-8
Source and lot/batch no.: Sigma Aldrich, lot n°STBJ4851
Active substance content: 99.9%
Expiry date of lot/batch: October 2022
Storage conditions: Ambient, dark.

Analysis parameters

Method type GC-MS (quantification method)
Instrument: Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column: Thames Restek, Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness)
Oven: 100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature: 280°C
Injection volume: 1 µL
Injector temperature: 250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas: Helium
Flow rate: 1.1 mL/min
Ionisation mode: Electron Impact Ionisation (EI)
Acquisition type: SIM (Selected Ion Monitoring)
Ion source temperature: 230°C
Quadrupole temperature: 150°C
Retention time: Thymol: approx. 15.8 min
Monitored ions (m/z): Thymol: 91 (30 ms dwell) for quantification.
Confirmation: see below
Method type GC-MS (confirmation method)
Instrument: Agilent Series 7890A Gas Chromatograph with 5975C and 7010B Network mass selective detector
Analytical column: Thames Restek, Rtx-1701 (30.0 m x 0.32 mm i.d., 1 µm film thickness)
Oven: 100°C (hold for 2 min) - ramp at 5°C/min - 160°C - ramp at 15°C/min - 250°C
Transfer line temperature: 280°C
Injection volume: 1 µL
Injector temperature: 250°C pulsed-splitless (pulse pressure 30 psi/2min)
Carrier gas: Helium
Flow rate: 1.1 mL/min
Ionisation mode: Electron Impact Ionisation (EI)
Acquisition type: SIM (Selected Ion Monitoring)
Ion source temperature: 230°C
Quadrupole temperature: 150°C
Retention time: Thymol: approx. 15.8 min
Monitored ions (m/z): Thymol: 91 (30 ms dwell), 150 (30 ms dwell), 115 (30 ms dwell)

Sample preparation

Extraction: An amount of 10 g ± 0.1 g of homogenised sample of matrix was weighed into a 50-mL centrifuge tube. Fortifications for preparation of procedural recoveries were performed at this step. For extraction, exactly 20 mL of acetonitrile were added, then the centrifuge tube was capped and shaken

vigorously by hand for a 30 s and vortex for 1 min. Thereafter, one citrate extraction tube containing 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, the sample tube was centrifuged for 5 min at 4000 rpm at 4°C.

Clean-up: All supernatant was transferred into a 50-mL centrifuge tube. One tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for a 30 s and vortex for 1 min, then the sample tube was centrifuged for 5 min at 4000 rpm at 4°C. 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube, 50 µL of toluene was added in each tube.

The supernatant was evaporated until 5.0 mL using a nitrogen evaporator set at 40°C. The two tubes were combined in the same 15-mL polypropylene tube and the combined supernatant was evaporated until 0.5 mL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 1.0 mL and the sample was vortexed during 5 minutes.

Final Fill-up for Analysis: The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

Stock solutions and calibration standards

Stock solution of thymol: the thymol reference item (between 2 – 50 mg) was dissolved in acetonitrile with the aid of an ultrasonic bath in order to obtain a stock solution of 1 000 µg/mL.

The stock solutions were further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and/or preparation of matrix matched calibration solutions. 9 mixed intermediate standard solutions of calibration standards were prepared spanning the concentration range 40 to 0.2 µg/mL in acetonitrile.

Calibration standard: Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions. 9 matrix-matched calibration standards were prepared spanning the concentration range 4 000 to 20 ng/mL in control extract or solvent (acetonitrile).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Accuracy and repeatability

Control (untreated) specimens of matrices of apple were fortified prior to extraction with the fortification solutions as described below. Two fortification solutions of concentrations 10.0 µg/mL and 1.0 µg/mL were prepared from the calibration stock solution and these solutions were used respectively to spike control apple samples level of 0.01 mg/kg and 0.1 mg/kg.

Calculations

Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was lower or equal to 25 %, otherwise a mathematical correction for the signal drift was applied according to the following equation:

Adjusted Analyte Residue Concentration (mg/kg) = Analyte Residue Concentration (mg/kg) x A/B

A = concentration of the standard used as bracketing standard in the initial calibration curve (mg/kg)

B = mean concentration of the two bracketing standards injected before and after the sample

A linear calibration function ($y = a \times x + b$) as determined by software MS Quantitative Analysis was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_{A_corr} - b}{a}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software MS Quantitative Analysis
Additionally, If	Only for recovery samples: Peak area of analyte in the final solution (counts) (y) corrected for (mean) peak

needed A_{A_corr}	area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	Slope of calibration curve (counts · mL/ng)
b	y -axis Intercept of the calibration curve (counts)

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{end} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation method for the thymol identification was not required as the GC-MS method is highly specific (three different ions were monitored).

Findings

Linearity:

Linearity was confirmed for the quantification ion (m/z 150) over the concentration range 20 ng/mL to 4 000 ng/mL, corresponding to sample concentrations of 0.0025 mg/kg to 0.5 mg/kg of thymol residues in apple and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract. The equation to the calibration line obtained with nine standards was $186x + 1035$, the correlation coefficient R was at least 0.9989 (y = peak area of thymol, x = concentration of thymol (in ng/mL)).

The linearity of the method is validated for this range of concentration of thymol.

No data was provided for the two confirmation ions monitored (m/z 150 and 115) or the second method proposed.

Specificity:

Specificity was studied by analysis samples of standards of thymol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with thymol, treated apple sample.

No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of thymol. In addition, validation was performed using matrix-matched standard. The method is considered specific.

Copies of relevant chromatograms are provided for standards of thymol, matrix-matched standards solutions for apple and apple bunches, and control (untreated) apple sample, control apple sample spiked with thymol, treated apple sample for the quantification ions (m/z 150).

In addition, a second method involving a different column was used to confirm analyte identification.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is 0.01 mg/kg in apple.

The limit of detection (LOD) was 0.0025 mg/kg (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of fortifications of control (untreated) test portions

of the apples for the quantification ions (m/z 150).

Fortification level (mg/kg)	Mean (%)	RSD (%)	n
0.01 (LOQ)	87	16	8
0.10	91	12	8
Overall	89	14	16

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 - 120%) and the RSD obtained is well below the limit value of 20%.

No data was provided for the two confirmation ions monitored (m/z 150 and 115) or the second method proposed.

Conclusions

Thymol is quantified after extraction from apple samples, by GC-MS (three ions monitored).

This analytical method for the determination of thymol content in apples samples has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in apples.

The method presented herewith is satisfactory and can be applied to quantify thymol in apples. However, the validation of the method for the two confirmation ions was not performed and no data was provided for these two ions.

Assessment and conclusion by applicant:

The validation of the method was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and was successfully validated. The method is acceptable for the quantification of thymol in apples.

KCP 5.2/05 (A 2.3.2.1/05 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in apples was not previously evaluated at EU level.</p> <p>The method was found to be valid according to the guidance document SANTE/2020/12830, rev.1 for the determination of eugenol, geraniol, thymol and methyl-eugenol in apples with the tested LOQ of 0.01 mg/kg.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for three (3) selected MS fragment ions for eugenol, geraniol, thymol and methyl-eugenol are within 70 – 110% with relative standard deviations ≤ 20% and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.</p> <p>The LOQ was successfully established at 0.01 mg/kg in apples for all three ions.</p> <p>The LOD was set at the level of the lowest acceptable calibration standard which is 0.0025 mg/kg.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/05 (A 2.3.2.1/05 of this dRR)
Report author	Driss F
Report year	2021c
Report title	Validation of residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in apple
Report No	S20-06529
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test	None

guideline

Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS and LC-MS/MS.

The method was used in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters

Method type GC-MS

Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)

Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)

Confirmation method: Rtx-1701 (30.0 m x 0.32 mm i.d., 1 µm film thickness, Thames Restek)

Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)

Injection mode Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz

Purge Flow 50 mL/min at 0.05 min

Injection volume 1 µL (depending on sensitivity)

Injector temperature 250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 16.0 min for thymol

Ionisation mode Electron Impact Ionisation (EI)

Scan type SIM (Selected Ion Monitoring)

Ion source temperature 230°C

Quadrupole temperature 150°C

Solvent delay 6 min

Ions monitored Quantification method Thymol: 91# (30 ms dwell)

Confirmation method Thymol: 150 (30 ms dwell), 115 (30 ms dwell)

dwel)

proposed/used for quantification but any of the ions listed can be used for quantification or qualification

Sample preparation

An amount of $10 \text{ g} \pm 0.1 \text{ g}$ of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO_4 , 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 μL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, eugenol concentration in the final extract was 0.1 $\mu\text{g/mL}$, and for samples fortified at 0.1 mg/kg, eugenol concentration in the final extract was 1.0 $\mu\text{g/mL}$.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 $\mu\text{g/mL}$. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 $\mu\text{g/mL}$ (equivalent to 0.0025 to 0.50 mg/kg of thymol in initial apples samples).

All solutions were stored at 1 °C to 10 °C (typically) in a brown glass flask in the dark.

Matrix effect

The effect of apples on the GC-MS and LC-MS/MS response was assessed by comparing mean peak areas of matrix-matched standards (90% matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [100 \times (A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \times C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extract(s)
$C_{\text{Solv-Std}}$	Nominal concentration of standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 70 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Extracts were quantified and stored for 15 days in the dark at 1 to 10°C and quantified again after storage. Results of both quantifications were compared to confirm extract stability. One MS fragment ion per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{[A_A \text{ or } A_{A_corr}] - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
A_{A_corr}	Only for recovery samples: Peak area of analyte in the final solution (counts) corrected for (mean) peak area of control sample = $A_A - (\text{mean}) \text{ blank peak area}$
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by using two separate GC columns and monitoring separate fragment ions/mass transitions: GC-MS with Rtx-wax column and fragment ion m/z 91 and GC-MS with Rtx-1701 column and ions m/z 150 and 115.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Thymol (%)		
		Quantification (<i>m/z</i> 91)	Confirmation (<i>m/z</i> 115)	Confirmation (<i>m/z</i> 150)
Apples (NEU)	80	(+) 3.7	-	-
Apples (SEU)	80	(+) 12.8	(+) 7.4	(+) 3.3

Matrix suppression or enhancement was $\leq 20\%$ in Apples for thymol and thus deemed to be insignificant. However, it was significant for eugenol and geraniol (see above sections for eugenol and geraniol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$ ($n = 8$), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial apple samples. Linearity was confirmed for each method:

- *m/z* 91: $y = 35x + 222$; $r = 0.9974$, $r^2 = 0.9949$
- *m/z* 115: $y = 151x - 1190$; $r = 0.9984$, $r^2 = 0.9968$
- *m/z* 150: $y = 71x - 25$; $r = 0.9992$, $r^2 = 0.9983$

Both methods are linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for both methods.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0025 mg/kg.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in apple matrix.

50 samples at LOQ and 50 samples at 10xLOQ were tested for apples because some background residues in the controls that might be quite variable were expected.

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 91 (Proposed for Quantification)							
Apples	0.01	90, 89, 102, 114, 112, 90, 83, 127, 141, 127, 124, 109, 128, 118, 116, 98, 78, 107, 115, 99, 100, 108, 139, 84, 84, 97, 115, 99, 98, 88, 95, 98, 101, 81, 80, 94, 73, 77, 78, 89, 78,88, 80, 100, 97, 77, 80, 92,72, 85	98	18	50	94	19
	0.1	110, 97, 121, 125, 114, 105, 111, 89, 102, 91, 112, 100, 106, 97, 103, 99, 106, 101, 95, 91, 93, 107, 111, 110, 68,69, 76,69, 69, 84, 69, 76, 77, 78,79, 74, 62, 71, 74, 78, 91, 92, 78, 59, 83, 84, 75, 70, 82	89	19	49 #		
Fragment <i>m/z</i> 115 (Proposed for Confirmation)							
Apples	0.01	88, 81, 83, 86, 75, 72, 97, 89, 77, 80, 73, 76, 77, 78, 72, 83, 90, 78, 86, 93, 82, 74, 95, 80, 83,	82	9	25*	80	9
	0.1	76, 77, 79, 78, 74, 85, 82, 75, 99, 88, 75, 70, 67, 70, 69, 68, 80, 88, 78, 86, 81, 80, 73, 75, 75	78	9	25*		
Fragment <i>m/z</i> 150 (Confirmation)							
Apples	0.01	85, 75, 77, 79, 71, 71, 80, 78,	76	7	25*	76	8

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
		68, 70, 71, 73, 75, 76, 75, 74, 79, 74, 84, 88, 79, 69, 89, 74, 78					
	0.1	73, 75, 76, 75, 73, 83, 80, 73, 96, 86, 73, 68, 65, 70, 68, 67, 77, 87, 76, 83, 80, 78, 71, 73, 73	76	9	25*		

Dixon test was used to exclude one value out of the range (around 600%)

* 25 samples were analysed for confirmation

No observable peak was detected in any control sample extract. Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-matched standards of thymol, control matrix, and control matrix spiked with thymol.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the thymol.

Blank correction was not performed. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for the ion and transition monitored. Moreover, mass spectra of thymol are provided to justify the choice of ion and ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within $\pm 10\%$ of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1 °C to 10 °C for 70 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Thymol was found to be stable in final extracts of apple for 15 days when stored at typically 1°C to 10°C in the dark.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 25) (%)	Rel. Std. Dev. (n = 25) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Thymol - Fragment m/z 91 (Proposed for Quantification)						
Apple	0	0.01	97, 115, 99, 98, 88, 95, 98, 101, 81, 80, 94, 73, 77, 78, 89, 78, 88, 80, 100, 97, 77, 80, 92, 72, 85	88	12	(-) 19
	15		60, 55, 77, 63, 62, 66, 76, 75, 70, 69, 65, 61, 77, 75, 77, 75, 73, 85, 69, 71, 73, 72, 75, 79, 70	71	10	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

Thymol is extracted from apple matrix using acetonitrile followed by solid-phase extraction clean-up, then quantified by GC-MS using two different columns and monitoring three fragment ions for confirmation of method specificity.

This analytical method for the determination of thymol content in apple matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in apples.

The method presented herewith is satisfactory and was applied to quantify thymol in apples in Eurofins study S20-06361 and S20-06527 in the same test facility (Eurofins Agrosience Services Chem SAS, France), which is included in this submission.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in apples was not previously evaluated at EU level. It was performed under GLP according to Guideline SANTE/2020/12830 rev.1 and was successfully validated.

The method is acceptable for the quantification of thymol in apples.

KCP 5.2/06 (A 2.3.2.1/06 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The study “<i>Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions</i>” was not previously evaluated at EU level.</p> <p>Sample extraction and determination of residues was performed according to an analytical procedure that was validated in study S20-06529. Quantification was performed by use of GC-MS detection.</p> <p>The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte.</p> <p>The recoveries for each of the analytes at each interval were within 70 – 110%, except at 30 days and 287 days, where the values were in the range 70-123%. The overall mean recoveries were in the range 70-110% and the deviations at 30 days and 287 days were considered not to affect the integrity of the results.</p> <p>The overall mean relative standard deviation covering all testing intervals was $\leq 20\%$ for all analytes.</p> <p>With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the storage samples.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/06 (A 2.3.2.1/06 of this dRR)
Report author	Driss F.
Report year	2021d
Report title	Storage Stability of Eugenol, Methyl Eugenol, Geraniol and Thymol in apple under Deep Frozen Conditions
Report No	S20-06527
Document No	Not applicable
Guidelines followed in study	SANTE/2020/12830 rev.1
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of apples were extracted with acetonitrile. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and aliquots were then evaporated after the addition of toluene. The extract was reconstituted with acetonitrile prior to injection on the GC-MS.

The method used in this study was validated within Study n°S20-06529 [Driss, F., 2021], included in this submission. Therefore, the method performance was verified in terms of selectivity, linearity, accuracy and precision.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS																
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)																
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)																
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)																
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min) Liner Topaz																
Purge Flow	50 mL/min at 0.05 min																
Injection volume	1 µL (depending on sensitivity)																
Injector temperature	250°C																
Column oven temperature programme	<table><tr><th>Step</th><th>Rate [°C/min]</th><th>Temperature [°C]</th><th>Hold time [min]</th></tr><tr><td>1</td><td>-</td><td>100</td><td>2.0</td></tr><tr><td>2</td><td>5.0</td><td>160</td><td>0.0</td></tr><tr><td>3</td><td>15</td><td>250</td><td>0.0</td></tr></table>	Step	Rate [°C/min]	Temperature [°C]	Hold time [min]	1	-	100	2.0	2	5.0	160	0.0	3	15	250	0.0
Step	Rate [°C/min]	Temperature [°C]	Hold time [min]														
1	-	100	2.0														
2	5.0	160	0.0														
3	15	250	0.0														
Retention time:	Approx. 16.0 min for thymol																
Ionisation mode	Electron Impact Ionisation (EI)																
Scan type	SIM (Selected Ion Monitoring)																
Ion source temperature	230°C																
Quadrupole temperature	150°C																
Solvent delay	6 min																
Ions monitored	Thymol: 91 (30 ms dwell) Since the method was already validated for specificity, this study only monitored a single ion.																

Sample preparation

An amount of 10 g ± 0.1 g of homogenised apple sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 5 minutes at 4°C.

Clean-up:

All supernatant was transferred into a 50-mL centrifuge tube and one tube of dSPE containing 1200 mg MgSO₄, 400 mg PSA and 400 mg GCB was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute, then centrifuged for 5 minutes at 4000 rpm at 4°C. The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and 8.0 mL of supernatant was transferred into one polypropylene tube and 8.0 mL was transferred in a second polypropylene tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. The contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.0 µg/mL.

Stock solutions and calibration standards

A mixed stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL for each analyte. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of apples which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/mL (equivalent to 0.0025 to 0.50 mg/kg of geraniol in initial apple samples).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Method performance

Selectivity was assessed by extracting and analysing a control sample according to the method to investigate the presence of residue or background interference at the retention time of the analytes.

Procedural recovery was determined by fortification of control samples with known amounts of geraniol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.1 mg/kg (10x LOQ). Analysis was performed by single extraction and single injection.

Since the method had already been validated, matrix effects were not investigated, and a single ion was monitored per analyte.

Calculations

The percentage of analyte level found in storage samples relative to the nominal fortification level (R_{Rel}), and procedural recoveries ($R_{ProcRec}$) are calculated as follows:

$R_{Rel} (\%)$ and $R_{ProcRec} (\%) =$	$\frac{R_A}{F} \times 100$
R_A	Unrounded residue level of analyte found in the sample (mg/kg)
F	Nominal sample fortification level (mg/kg)

The percentage of found analyte corrected for the procedural recovery of the individual date of extraction ($P_{Corrected}$) is calculated as follows:

$R_{Corrected} (\%) =$	$\frac{R_{Rel_Mean}}{R_{ProcRec_Mean}} \times 100$
R_{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
$R_{ProcRec_Mean}$	Unrounded procedural recovery of the individual date of extraction (%)

The percentage of remaining analyte found in the stored samples relative to the mean residues of day 0 analysis ($P_{Remaining}$) is calculated as follows:

R _{Remaining} (%)=	$\frac{R_{Rel_Mean}}{R_{Day0_Mean}} \times 100$
R _{Rel_Mean}	Unrounded mean percentage of analyte level found in storage samples (%)
R _{Day0_Mean}	Unrounded mean R _{Rel} for Day 0 analysis (%)

Findings

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL (n = 8), corresponding to analyte concentrations of 0.0025 mg/kg to 0.5 mg/kg in initial apple samples. Linearity was confirmed for a single ion since the method was already separately validated for three ions:

Thymol	m/z 91: y = 228x + 2546; r = 0.9981, r ² = 0.9963
--------	--

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg.

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.002 mg/kg.

Accuracy, Repeatability (precision):

The recoveries of the day 0 storage samples and freshly fortified procedural recovery samples document the analytical performance in terms of accuracy and repeatability throughout the study. Fortification level was at 10x LOQ with analytes fortified jointly for procedural recoveries and fortified separately for day 0 storage samples. Procedural recoveries were handled and stored in the same way and for the same time period as the extracts of the storage samples that were prepared within the same analytical set. The following recoveries were obtained:

Matrix	Fortification level (mg/kg)	Recovery (%)						Mean (%)	Rel. Std. Dev. (%)
		0 days	30 days	112 days	161 days	285 days	287 days		
	Thymol								
Apple	0.1	90, 91, 87	118	81	109	108	123*	101	15

* Reanalysis performed to confirm results obtained at 285 days

Selectivity:

One control sample was extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analytes for each analytical set. The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Example chromatograms for each matrix and analytes representing control samples and samples fortified at 10x LOQ level are presented in the report.

Extraction efficiency:

Not needed to be demonstrated as part of this study.

Conclusions

The method validated as part of Study n°S20-06529 for the quantification of residues of thymol in apple matrix was used to assess the stability of residues of thymol in apples. Selectivity, linearity and procedural recoveries were verified and found to be acceptable.

The method presented herewith is satisfactory and was applied to quantify thymol in apples.

Assessment and conclusion by applicant:

The method used to verify the stability of thymol residue in apples was confirmed to be acceptable for its purpose. The method was separately validated in Study n°S20-06529 and verified as part of this study. It

was performed under GLP according to Guidelines SANTE/2020/12830 rev.1.
The method is acceptable for the quantification of thymol in apples.

TOMATO, CUCUMBER AND STRAWBERRY

KCA 4.2/01 (A 2.3.2.1/07 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the methods for analysis for thymol in tomato, cucumber and strawberry was not previously evaluated at EU level.</p> <p>The analytical method was been validated for the determination of geraniol and thymol in tomato, cucumber and strawberry with the tested LOQ of 0.05 mg/kg according to the guidance documents SANCO/825/00 rev. 8. 1 and SANCO/3029/99.</p> <p>LOQ of 0.05 mg/kg was established for geraniol and thymol in tomato, cucumber and strawberry.</p> <p>For tomato and strawberry, five recovery determinations each were performed at the levels of 0.05 mg/kg (LOQ) and 0.50 mg/kg, representing full validation sets according to the guidance documents SANCO/825/00 rev. 8.1 and SANCO/3029/99.</p> <p>For cucumber, only a reduced validation set with each three recovery determinations at the levels of 0.05 mg/kg (LOQ) and 0.50 mg/kg was performed, since cucumber belongs to the same crop group as tomato(= high water) and therefore a reduced validation is sufficient according to SANCO/3029/99.</p> <p>The mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CA 4.2/01 (A 2.3.2.1/07 of this dRR)
Report author	Wiesner F., Breyer N.
Report year	2017
Report title	Validation of an Analytical Method for the Determination of Residues of Geraniol and Thymol in Tomato, Cucumber and Strawberry
Report No	S16-03357
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1
Deviations from current test guideline	None
Previous evaluation	No, not already submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is quantified in tomatoes, cucumber and strawberries using LC-MS/MS and a multi-residue method based on QuEChERS⁹. Various extraction techniques were used that are described below. Although the study report refers to thymol and geraniol, only thymol is considered in this summary.

Materials and methods

⁹ EN 1 5662:2008 "Foods of plant origin - Determination of pesticide residues using GC-MS and/or LCMS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE – QuEChERS Method"; German version (2009).

Analytical grade thymol was used as test item

Test material

Name: Thymol analytical grade
CAS number: 89-83-8
Source and lot/batch no.: Dr. Ehrenstorfer, Lot 40930
Active substance content: 99.0% (equivalent to 990 g/kg)
Expiry date of lot/batch: 07 October 2020
Storage conditions: $\leq -18^{\circ}\text{C}$, dark

Test system

Tomato (fruit, high water)
Cucumber (fruit, high water)
Strawberry (fruit, high acid)

Analysis parameters

Strawberries

Method type LC-MS/MS
Instrument: 1290 Binary Rapid Resolution LC System, Agilent Technologies
API 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Analytical column: Luna Phenyl-Hexyl, 100 mm x 4.6 mm, 3 μm , Phenomenex
Column temperature: 60°C
Injection volume: 40 μL
Mobile phase: Eluant A: Acetonitrile; Eluent B: Water + 5 mM ammonium acetate
Gradient:

Time (min)	Eluent A	Eluent B
1.0	50	50
1.0 – 6.0	50 \rightarrow 90	50 \rightarrow 10
6.0 – 6.1	90 \rightarrow 95	10 \rightarrow 5
6.1 – 7.0	95	5
7.0 – 7.1	95 \rightarrow 50	5 \rightarrow 50
7.1 – 10	50	50

Flow rate: 0.6 mL/min
Ionisation type: ESI, Turbolon spray
Polarity: Negative
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)
Retention time: approx. 4.6 min
Monitored ions/transitions Thymol: 149 \rightarrow 134 (quantification)
149 \rightarrow 133 (confirmation)

Tomatoes and cucumbers

Method type LC-MS/MS
Instrument: 1290 Binary Rapid Resolution LC System, Agilent Technologies
API 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Analytical column: Luna Phenyl-Hexyl, 100 mm x 4.6 mm, 3 μm , Phenomenex
Column temperature: 30°C
Injection volume: 40 μL
Mobile phase: Eluant A: Acetonitrile; Eluent B: Water + 5 mM ammonium acetate
Gradient:

Time (min)	Eluent A	Eluent B
0.00 – 3.00	25 \rightarrow 95	75 \rightarrow 5
3.0 – 6.00	95	5
6.00 – 6.01	95 \rightarrow 25	5 \rightarrow 75
6.01 – 9.00	25	75

Flow rate: 0.6 mL/min

Ionisation type:	ESI, Turbolon spray
Polarity:	Negative
Scan type:	MS/MS, Multiple Reaction Monitoring (MRM)
Retention time:	approx. 4.6 min
Monitored ions/transitions	Thymol: 149 → 134 (quantification) 149 → 133 (confirmation)

Sample preparation

Where necessary, samples are fortified prior to extraction. The solvent is allowed to evaporate before starting the extraction procedure.

Extraction and liquid/liquid partition:

Homogenized specimen (10 g) are weighed into 50 mL Sarsedt centrifuge tubes. 10 mL of acetonitrile (V_{EX}) is added to the tube which is capped and shaken for 15 minutes using a platform shaker. Then 4 g magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate are added, the centrifuge tube is capped and immediately shaken by hand or using a vortex for about 2 minutes. After shaking, the sample tube is centrifuged for 5 minutes at 4000 rpm.

SPE clean-up:

6 mL of the upper acetonitrile phase are diluted with 54 mL ultra-pure water. The SPE cartridge is conditioned using 1 x 5 mL acetonitrile and 1 x 5 mL ultra-pure water. The sample solution (60 mL) is transferred to the cartridge and allowed to percolate through under gravity. The eluate is discarded. A test tube is placed under the cartridge and 6 mL of acetonitrile are passed through the cartridge without vacuum.

For tomatoes and cucumbers, 800 μ L of the eluate are mixed with 200 μ L ultra-pure water, mixed well and transferred to vials for quantification by LC-MS/MS.

For strawberries, 800 μ L of the eluate are mixed with 200 μ L of 0.5% acetic acid, mixed well and transferred to vials for quantification by LC-MS/MS.

Stock solutions, calibration standards, fortification solutions

A stock solution of concentration 400 μ g/mL was prepared by accurately weighing 10.0 mg or test item into a 25 mL volumetric flask, dissolving and adjusting to volume with acetone.

A fortification solution of concentration 50 μ g/mL is prepared from the stock solution by dilution in acetonitrile. An aliquot of this solution is further diluted in acetonitrile to produce a second fortification solution of concentration 5.0 μ g/mL.

Calibration standards in acetonitrile: water (80:20 v/v) are prepared by sequential dilution of the stock solution in acetone. The calibration range covers the concentration range 12 – 6000 ng/mL and includes 10 standards.

Calibration standards were also prepared in acetonitrile: 0.5% acetic acid (80:20 v/v). They were prepared by sequential dilution of the stock solution in acetone and span the concentration range 12 – 6000 ng/mL. This calibration range includes 10 standards.

Investigation of matrix effects showed no matrix effect was observed for thymol. Therefore, solvent standards were used for all matrices.

At least 7 standards are used for each calibration.

Accuracy (recovery) samples

Recovery samples are prepared at two fortification levels, 0.05 mg/kg and 0.5 mg/kg in each matrix (strawberry, cucumber and tomato). Samples are homogenized, fortified and the solvent is allowed to evaporate prior to extraction. Samples are then prepared according to the procedure described under Sample preparation above.

Following extraction and clean-up, a 0.05 mg/kg fortified sample yield an extract of concentration 40 ng/mL, while a 0.5 mg/kg fortified sample yields a 400 ng/mL extract.

Calculations

The evaluation of the results was based on the average response factor which was calculated from the calibration standards. At least 7 external standard solutions were used for the determination of the average response factor.

The residues (R) in mg/kg were calculated according to the following equation:

$$R = \frac{A_A \times V_{EX} \times V_{End}}{AvF \times G \times V_{Ali} \times CF} \times DF$$

Where:

R = Residues of the analyte in mg/kg

A_A = Peak area of the analyte in the sample solution in counts

AvF = Average response factor

The average response factor is calculated as follows:

$$AvF = \frac{A_{St1}/C_{St1} + A_{St2}/C_{St2} + \dots + A_{StN}/C_{StN}}{N}$$

C_{st} = Concentration of external standard solution in ng/mL

A_{st} = Peak area in the external standard solution in counts

N = Number of external standard solutions

V_{Ex} = Extraction volume: 10 mL

V_{Ali} = Aliquot volume: 0.80 mL

V_{End} = Final volume: 1.0 mL

G = Sample weight of the analytical specimen: 10 g

CF = Conversion factor for ng into µg (= 1000)

DF = Dilution factor (1 = no dilution)

Percent recovery from fortified specimen was calculated using the following expressions:

$$Recovery (\%) = \frac{R_{fortified}}{F} \times 100$$

Where:

R_{fortified} = Residues of fortified specimen, in mg/kg

F = Fortification in mg/kg

Findings

Linearity:

Tomato and Cucumber 149 → 134 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 852.893x – 132.4431 Coefficient of determination R ² : 0.9991 Coefficient of correlation r: 0.9996
Tomato and Cucumber 149 → 133 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 409.6994x – 1387.3685 Coefficient of determination R ² : 0.9985 Coefficient of correlation r: 0.9993
Tomato and Cucumber (reinjection) 149 → 134 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 8446425 – 6654.6159 Coefficient of determination R ² : 0.9972 Coefficient of correlation r: 0.9986
Strawberry 149 → 134 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 277.2724x + 48.1154 Coefficient of determination R ² : 0.9997 Coefficient of correlation r: 0.9986
Strawberry 149 → 133 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 128.7328x – 187.6406 Coefficient of determination R ² : 0.9982 Coefficient of correlation r: 0.9991
Strawberry (reinjection) 149 → 134 m/z	Linear over 12 to 600 ng/mL (n = 7) Equation to the calibration line: 339.6348x + 454.0548 Coefficient of determination R ² : 0.9998 Coefficient of correlation r: 0.9999

Specificity:

LC-MS/MS determination was conducted by monitoring two mass transitions for thymol (149→134 m/z and 149→133 m/z). Due to enhanced sensitivity mass transition 149→134 m/z is proposed to be used for quantification, but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two control samples for tomato and strawberry were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For the reduced validation set of cucumber, one control only was extracted and analysed. For both mass transitions, the samples showed no significant interference (above 30% of LOQ) at the retention time of the analyte in any investigated matrix, therefore showing that the method is highly specific.

Blank correction was not performed.

Copies of relevant chromatograms for both thymol mass transition (149→134 m/z and 149→133 m/z) are provided for solvent standard solution, untreated specimen of each matrix (strawberry, cucumber and tomato), specimen spiked with thymol of each matrices (strawberry, cucumber and tomato).

Moreover, MS/MS spectrum (initial product ion scan) of Thymol ion [M+H]⁺ (149 m/z) are provided and confirmed the selected mass transition 149→134 m/z and 149→133 m/z for the analyte.

LOD, LOQ:

The LOQ of the method is defined as the lowest analyte concentration at which the methodology had been successfully validated. Although considerable efforts were made to meet the recommended LOQ of 0.01 mg/kg, this was not achievable taking into account the available instrumentation and the nature of the analytes. Therefore, during this validation an LOQ of 0.05 mg/kg was established for thymol in tomato, cucumber and strawberry.

The LOD was set at 30% of the LOQ, which is 0.015 mg/kg. Peaks at the LOD were equivalent to or more than three times the background noise.

Accuracy, Repeatability (precision):

Crop, transition	Fortification	Mean Recovery	% RSD	Horrat value
Tomato 149 → 134 m/z	0.05 mg/kg	98%	5.0%, n = 5	0.30
	0.5 mg/kg	102%	3.0%, n = 5	0.25
	Combined	100%	4.4%, n = 10	-
Tomato 149 → 133 m/z	0.05 mg/kg	89%	6.6%, n = 5	0.39
	0.5 mg/kg	96%	1.8%, n = 5	0.15
	Combined	93%	5.7%, n = 10	-
Cucumber 149 → 134 m/z	0.05 mg/kg	102%	7.4%, n = 3	0.44
	0.5 mg/kg	101%	6.6%, n = 3	0.55
	Combined	102%	6.3%, n = 6	-
Cucumber 149 → 133 m/z	0.05 mg/kg	84%	5.5%, n = 3	0.33
	0.5 mg/kg	88%	2.3%, n = 3	0.19
	Combined	86%	4.9%, n = 6	-
Strawberry 149 → 134 m/z	0.05 mg/kg	102%	7.0%, n = 5	0.42
	0.5 mg/kg	95%	3.6%, n = 5	0.30
	Combined	98%	6.7%, n = 10	-
Strawberry 149 → 133 m/z	0.05 mg/kg	95%	9.9%, n = 5	0.59
	0.5 mg/kg	91%	3.4%, n = 5	0.29
	Combined	93%	7.8%, n = 10	-

The data indicates that the accuracy and precision of the method is acceptable for all test matrices (tomato, cucumber and strawberry) for both thymol mass transition (149→134 m/z and 149→133 m/z), in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Solvent calibration solutions were found to be stable for 19 days when stored at 1 – 10°C in the dark.

Extracts were found to be stable for at least 16 days for tomato and 10 days for strawberry when stored at 1°C to 10°C in the dark.

Conclusions

Thymol is extracted in tomatoes, cucumber and strawberries with various extraction techniques, and then quantified by LC-MS/MS (two ions monitored). Propofol was used as internal standard.

Thymol is quantified in tomatoes, cucumber and strawberries using LC-MS/MS and a multi-residue method based on QuEChERS. Various extraction techniques were used.

Thymol was successfully quantified in tomato, cucumber and strawberry and the methods proposed have been fully validated. Specificity of the method and absence of interference from the test item, internal standard or the various matrices was demonstrated, moreover two mass transition were followed and validated to confirm the identity of thymol. The Limit of Quantification was 0.05 mg/kg (corresponding to 0.05 ppm) for thymol in each matrix (tomatoes, cucumber and strawberries).

The methods presented herewith are satisfactory and can be applied to quantify thymol residues in the described matrices (high water and high acid fruits).

Assessment and conclusion by applicant:

The validation of the methods for analysis for thymol in tomato, cucumber and strawberry was not previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3029/99 rev.4 and was successfully validated.

The methods are acceptable for the quantification of thymol in tomato, cucumber (high water fruits) and strawberry (high acid fruits).

A 2.3.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

KCP 5.2/08 (A 2.3.2.2/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in body tissue (meat and liver) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination eugenol, methyl-eugenol, thymol and geraniol from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4.</p> <p>The LOQ is the lowest validated fortification level for eugenol, methyl-eugenol, thymol and geraniol and was thus successfully established at 0.01 mg/kg in meat and liver for all selected MS fragment ions or mass transitions.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations $\leq 20\%$ and thereby comply with the standard acceptance criteria of the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/08 (A 2.3.2.2/01 of this dRR)
Report author	Driss F
Report year	2021f
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body tissue (meat and liver)
Report No	S20-06625
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17

Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters for meat and liver (quantification method)

Method type	GC-MS
Instrument:	Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column:	Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas	Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode	Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz
Purge Flow	50 mL/min at 0.05 min
Injection volume	1 µL (depending on sensitivity)
Injector temperature	250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time:	Approx. 15.8 min for Thymol
Ionisation mode	Electron Impact Ionisation (EI)
Scan type	SIM (Selected Ion Monitoring)
Ion source temperature	230°C
Quadrupole temperature	150°C
Solvent delay	6 min
Ions monitored	Thymol for meat: 115# (100 ms dwell), 150 (100 ms dwell), 91 (100 ms dwell) Thymol for liver: 150 # (100 ms dwell), 115 (100 ms dwell)

proposed (and/or used) for quantification but both of the mass transitions listed can be used for quantification

Analysis parameters for liver (confirmation method)

Method type	LC-MS/MS
HPLC system	LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar) LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column	Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure	200 bar
Autosampler temperature	4°C
Column oven temperature	60°C
Injection volume	10 µL (depending on sensitivity)
Mobile phases	Eluent A: Acetonitrile; Eluent B: Water containing 5mM of ammonium acetate

Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0.0	25	75	0.6
	0.5	25	75	0.6
	3.0	95	5.0	0.6
	4.5	95	5.0	0.6
	5.5	25	75	0.6
	7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste

Retention time(s) Approx. 3.02 min for Thymol

Mass spectrometric conditions

MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer) SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)
Polarity	Negative ion mode for Thymol
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)
Capillary voltage (IS)	-4500 V (thymol)
Ionspray turbo heater (TEM)	100°C

Curtain gas (CUR)	Nitrogen set at [25-40] psi		Gas flow 1 (GS1)		Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)	
Collision gas (CAD)	Nitrogen set at 8 psi		Gas flow 2 (GS2)		Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)	
Analyte monitored	Mass transition monitored (<i>m/z</i>)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Thymol	149 → 134 (neg)	-85	-10	-20	-15	35

Sample preparation

Meat: An amount of 10 g ± 0.1 g of homogenised meat sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The

contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube is then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. the contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.0 µg/mL.

Liver: An amount of 10 g ± 0.1 g of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 µL of toluene is added; the supernatant was evaporated to 600 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 µL.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.125 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.25 µg/mL.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/ mL (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effects

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
A _{Solv-Std}	Mean peak area of solvent standard
A _{Matrix-Std}	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
C _{Solv-Std}	Nominal concentration of solvent standard in ng/mL
C _{Matrix-Std}	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 70 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into μg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ion transitions by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Thymol (%)		
Meat	80	<i>m/z</i> 115	<i>m/z</i> 150	<i>m/z</i> 91
		66.4	2.1	21.6
Liver	80	<i>m/z</i> 150	<i>m/z</i> 115	<i>m/z</i> 149→134
		6.5	10.7	5.4

Matrix effects were $\geq \pm 20\%$ and deemed to be significant in meat. For liver, it has been shown to be significant for geraniol (See above section for geraniol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL, corresponding to analyte concentrations of 0.025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver. Linearity was confirmed for each ion and method.

	Meat	Liver
<i>m/z</i> 91 (GC-MS)	$y = 281x + 813$ $r = 0.9991, r^2 = 0.9983$	--
<i>m/z</i> 150 (GC-MS)	$y = 311x + 2761$ $r = 0.9992, r^2 = 0.9985$	$y = 1473x + 9759$ $r = 0.9982, r^2 = 0.9964$
<i>m/z</i> 115 (GC-MS)	$y = 209x + 1528$ $r = 0.9994, r^2 = 0.9988$	$y = 744x + 18397$ $r = 0.9984, r^2 = 0.9967$
<i>m/z</i> 149→134 (LC-MS/MS)	--	$y = 195x - 386$ $r = 0.9998, r^2 = 0.9997$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for meat and liver, for both methods

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg in meat and 0.0027 mg/kg in liver.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 91							
Meat	0.1	81, 93, 83, 72, 91	84	10	5	86	11
	0.01	95, 99, 71, 89, 88	88	12	5		
Fragment <i>m/z</i> 150							
Meat	0.01	75, 77, 82, 73, 86	79	7	5	83	11
	0.1	93, 97, 71, 91, 87	88	11	5		
Fragment <i>m/z</i> 115							
Meat	0.01	93, 100, 96, 79, 103	94	10	5	91	11
	0.1	94, 99, 71, 91, 88	89	12	5		

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 150							
Liver	0.01	87; 87; 74; 85; 77	82	7	5	91	12
	0.1	104; 95; 100; 100; 104	101	4	5		
Fragment m/z 115							
Liver	0.01	97; 91; 73; 82; 82	85	11	5	93	11
	0.1	103; 97; 103; 98; 103	101	3	5		

Transition m/z 149→134							
Liver	0.01	67; 74; 81; 79; 90	78	11	5	83	9
	0.1	81; 89; 91; 85; 88	87	4	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of thymol, control matrix, and control matrix spiked with thymol. Two fragment ions were monitored by GS/MS and one selected mass transition was monitored by LC-MS/MS.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of thymol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix meat and liver, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 70 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for thymol re-analysed extracts were in the range 70-110% and ± 20 % of the original result for liver but not for meat. For liver, as the acceptance criteria were not fulfilled for geraniol (see above section for geraniol), final extracts of meat and liver are considered to be unstable for 17 and 8 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Thymol - Fragment m/z 150						
Meat	0	0.01	75; 77; 82; 73; 86	79	7	(+)30
	17		108; 99; 108; 92; 107	103	7	
Liver	0	0.01	87; 87; 74; 85; 77	82	7	(+)18
	8		100; 98; 83; 104; 98	97	8	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Thymol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for meat samples and quantified by GC-MS using two separate ions and by LC-MS/MS following one ion transition for liver samples. The method is specific.

This analytical method for the determination of thymol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and

SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify thymol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of thymol in meat and liver.

A 2.3.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

The DT₉₀ of thymol in soil is less than three days and therefore a method for the quantification of thymol in soil is not required.

A 2.3.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

KCP 5.2/09-(A 2.3.2.4/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in surface water was not previously evaluated at EU level.</p> <p>The methods were successfully validated and is considered suitable for the determination of residues of thymol, methyl-eugenol, geraniol and eugenol in surface water with an LOQ of 0.1 µg/L. Any of the three ions validated for thymol, geraniol and methyl-eugenol are suitable for quantification and/or confirmation. For eugenol, either of the two HPLC columns are suitable for quantification and/or confirmation.</p> <p>The LOQ was 0.1 µg/L.</p> <p>No significant matrix effects were observed therefore calibration solutions for thymol, methyl-eugenol and geraniol were prepared in solvent.</p> <p>All mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for methyl-eugenol, thymol and geraniol are within 70 – 120% with relative standard deviations ≤ 20%.</p> <p>Satisfactory accuracy and precision results were achieved for eugenol on two HPLC columns of differing chemistry (C₁₈ and pentafluorophenyl), monitoring a single transition; mean recovery values at each fortification levels of 0.1 µg/L and 1 µg/L for eugenol, are within 70 – 120% with RSD ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/09 (A 2.3.2.4/01 of this dRR)
Report author	Chambers J.
Report year	2020a
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in Surface Water
Report No	GW/19/001
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Thymol is extracted from surface water via steam distillation and quantified by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item

Test material

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBH8227
Active substance content:	99.9%
Expiry date of lot/batch:	September 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 20°C/min to 250°C
Transfer line temperature:	280°C
Injection volume	2 µL
Injector inlet temperature:	250°C Splitless
Carrier gas:	Helium
Flow rate:	2.6 mL/min
Acquisition type:	SIM
Retention time:	Thymol: approx. 7.4 min
Monitored ions	Thymol: 115, 135 and 150 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

500 mL surface water were transferred to a 500 mL round bottom flask. After fortification, anti-bumping granules were added and 10 mL sample transferred to a liquid-liquid extractor followed by 5 mL of hexane/ethyl acetate (1:1 v/v). The flask was attached to the liquid-liquid extractor, placed on a heating mantle, connected to a condenser and switched on. After the sample reached boiling point and when the vapour was observed to be condensing above the liquid-liquid extractor, the procedure ran for 90 minutes. After cooling, the upper organic layer was transferred by pipette to a graduated tube and 1 mL trimethylpentane added.

The remaining contents of the liquid-liquid extractor were transferred to a second graduated tube and partitioned with 2 mL hexane/ethyl acetate (1:1 v/v) and the upper organic layer was combined with the first. The tube was placed a dri-block and evaporated until < 1 mL remained. The contents were quantitatively transferred to a 1.0 mL volumetric flask and made up to the mark with hexane/ethyl acetate (1:1 v/v). An aliquot was transferred to an auto-sampler vial for determination by GC-MS.

For recovery samples fortified at 0.1 µg/L and 1.0 µg/L, final extract concentrations are 50 ng/mL and 500 ng/mL, respectively.

Stock solutions and calibration standards

Thymol Standard Solution: Thymol, 5 mg is accurately weighed and dissolved in acetone to produce a 1000 µg/mL stock solution. This stock solution is used to prepare 8 calibration standards spanning the concentration range 10 to 1500 ng/mL in trimethyl pentane.

No significant matrix effects were observed therefore calibration solutions for Thymol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 1 µg/mL and 10 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile. These fortification solutions are used to produce samples fortified at 0.1 µg/L and 1.0 µg/L. Fortified samples are then extracted and processed as described in sample preparation above. Final extract concentrations are 50 ng/mL and 500 ng/mL for 0.1 µg/L and 1.0 µg/L fortifications, respectively.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a $1/x$ weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f}{m \times V_s} \mu\text{g/L}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

V_s = sample volume

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/L}]}{\text{Amount spiked } [\mu\text{g/L}]} \times 100$$

Three ions were selected for thymol.

Confirmation of substance identification

Confirmation method for the thymol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 115, 135, 150) over the concentration range 10 ng/mL to 1000 ng/mL, corresponding to sample concentrations of 0.02 µg/L to 2.0 µg/L; seven standards were quantified:

115 m/z	135 m/z	150 m/z
Y = 34.79371x + 84.75949 R ² = 0.99981 r = 0.99990	Y = 228.92849x + 23.26414 R ² = 0.99986 r = 0.99993	Y = 64.209x + 42.42701 R ² = 0.9982 r = 0.9991

The linearity of the method is considered validated for this range of concentration of thymol.

Specificity:

Specificity of the analytical method was provided by the analysis of control matrix samples by GC-MS while monitoring three ions per analyte. In addition, validation was performed using analytical grade test material, precluding the need for further verification of substance identity. No interference or contamination peak was detected in control samples above 30% LOQ at the same retention time of Thymol. The method is specific.

Copies of relevant chromatograms are provided for standards of thymol, control surface water, and control surface water spiked with thymol for each ion monitored. Moreover a mass spectrum of thymol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ is

0.1 µg/L.

The limit of detection (LOD) was 0.02 µg/L (lowest calibration level).

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels in surface water and three ions were monitored.

	Thymol m/z 115			Thymol m/z 135			Thymol m/z 150		
Spike (µg/L)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.1	101	11.5	5	89	7.6	5	89	10.2	5
1	85	5.9	9	85	5.3	9	84	5.7	9
Overall	91	11.9	14	86	6.5	14	86	7.7	14

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Stability:

Stability of the analyte in solvent and matrix was determined. Thymol was found stable in solvent for 55 days, and thymol extracts were found to be stable for 22 days when stored at 4-6°C.

Conclusions

Thymol is extracted in surface water via steam distillation and quantified by GC-MS (three ions monitored).

Thymol was successfully quantified in surface water and the method proposed has been fully validated. Specificity of the method and absence of interference from the test item, internal standard or surface water was demonstrated. The Limit of Quantification was 0.1 µg/L for thymol in surface water.

The method presented herewith is satisfactory and can be applied to quantify thymol in surface water.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in surface water was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of thymol in surface water.

A 2.3.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

KCP 5.2/10 (A 2.3.2.5/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in air sampling cartridges was not previously evaluated at EU level.</p> <p>The method was successfully validated and is considered suitable for the determination of residues of thymol, eugenol, methyl-eugenol and geraniol in air with an LOQ of 1.2 µg/m³ according to guidance documents SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours.</p> <p>All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/10 (A 2.3.2.5/01 of this dRR)
Report author	Chambers J.
Report year	2020b
Report title	Thymol, Eugenol, Methyl Eugenol and Geraniol: Method Validation in

	Air
Report No	TS/19/003
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Residues of Thymol were extracted from pre-packed XAD-2 cartridges by sonication with ethyl acetate and final determination was performed by GC-MS, monitoring three ions of $m/z > 100$.

Although the study report refers to thymol, geraniol, eugenol and methyleugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item

Test material

Name:	Thymol analytical grade
Source and lot/batch no.:	Sigma Aldrich, batch number STBH8227
CAS number:	89-83-8
Active substance content:	99.9%
Expiry date of lot/batch:	September 2021
Storage conditions:	Ambient

Analysis parameters

Method type	GC-MS
Instrument:	Agilent 6980 GC and 5975 MSD
Analytical column:	Agilent CP-FFAP CB with 4 mm splitless single taper with glass-wool liner
Oven:	90°C initial temperature, hold for 2 minutes, ramp at 12.5°C/min to 215°C and hold for 1 minute
Transfer line temperature:	280°C
Injection volume	3 µL
Injector inlet temperature:	280°C pulsed-splitless
Carrier gas:	Helium
Flow rate:	2.0 mL/min
Acquisition type:	SIM
Retention time:	Thymol: approx. 9.5 min
Monitored ions	Thymol: 115, 135 and 150 m/z

Sample preparation

Where necessary, control matrix is fortified prior to sample treatment.

If the entire contents of the cartridge are required for analysis, transfer the front and rear segments of the cartridge to a screw-top vial including any glass-wool dividers.

If the front and rear segments of the cartridge are to be analysed separately, transfer the front segment of the cartridge to a screw-top vial including any glass-wool dividers before and after the sorbent, and transfer the rear segments of the cartridge to a screw-top vial including any glass-wool dividers after the sorbent, taking care not to pass the rear section through the front section to avoid contamination.

Add ethyl acetate (5 x 1 mL) extraction solvent to the empty cartridge, collecting all washes into the

(front segment) vial. Vortex the sample for 1 minute, sonicate the sample for 10 minutes and vortex the sample for 30 seconds. Transfer an aliquot into an auto-sampler vial and quantify by GC-MS. For recovery samples fortified at 0.5 µg/cartridge and 5.0 µg/cartridge, final extract concentrations are 100 ng/mL and 1000 ng/mL, respectively.

Stock solutions and calibration standards

Thymol Standard Solution: Thymol, 5 mg, is accurately weighed and dissolved in acetonitrile to produce a 1000 µg/mL stock solution. This stock solution is further diluted in ethyl acetate to produce an intermediate stock from which calibration standards in ethyl acetate are produced. The calibration solutions span the concentration range 20 to 1300 ng/mL in ethyl acetate (corresponding to 0.24 to 15.6 µg/m³).

No significant matrix effects were observed therefore calibration solutions for thymol were prepared in solvent.

Accuracy (recovery) samples

Two fortification solutions, of concentrations 200 µg/mL and 20 µg/mL are prepared from the calibration stock solution. Fortification solutions are prepared in acetonitrile.

The target LOQ for each analyte in air was 1.2 µg/m³. The limit of quantification (LOQ) was taken as the lowest fortification level where an acceptable mean recovery is obtained in the range 70 to 110% with an RSD < 20%.

The rate of air flow was 1.0 L/min for a total duration of 7 hours. This resulted in a volume of 420 L or 0.42 m³ over the 420-minute period, equating to a fortification level of 0.504 µg/cartridge, rounded to 0.50 µg/cartridge (0.42 m³ x 1.2 µg/m³).

The two ends of each cartridge were broken off using a tube cutter. The front segment of each cartridge was fortified at 0.5 µg/cartridge (LOQ) by pipetting 25 µl of the 20 µg/mL fortification solution and at 5 µg/cartridge (10 x LOQ) by pipetting 25 µl of the 200 µg/mL fortification solution.

Calculations

A multi-level calibration curve of the form $y = mx + c$ was obtained. The calibration curve was constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression using a 1/x weighting factor. The quantification of analyte in the final sample extract was made by comparison to the calibration curve of the form $y = mx + c$.

The concentration of in the sample extract was calculated as follows:

$$\text{Concentration (ng/mL)} = \frac{(A - b) \times V_f \times D}{m \times 1000} \mu\text{g/cartridge}$$

Where:

A = peak area

m = slope of the calibration curve

b = intercept of the calibration curve

V_f = final volume of the sample solution

D = Dilution factor

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found } [\mu\text{g/cartridge}]}{\text{Amount spiked } [\mu\text{g/cartridge}]} \times 100$$

Three ions were selected for Thymol.

Confirmation of substance identification

Confirmation method for the thymol identification was not required as the GC-MS method is highly specific (three ions are monitored).

Findings

Linearity:

Linearity was confirmed for three ions (m/z 115, 135, 150) over the concentration range 20 ng/mL to 1300 ng/mL, corresponding to sample concentrations of 0.24 µg/m³ to 15.6 µg/m³; eight standards were quantified:

115 m/z	135 m/z	150 m/z
Y = 57.25204x -11878432 R ² = 0.99989 r = 0.99994	Y = 387.51717x -1154.14698 R ² = 0.99991 r = 0.99995	Y = 112.41378x -319.47793 R ² = 0.99985 r = 0.99992

The linearity of the method is acceptable for this range of concentration of thymol.

Specificity:

Specificity of the analytical method was provided by the analysis of control matrix samples by GC-MS while monitoring three ions above m/z 100. No interference or contamination peak was detected above 30% LOQ at the same retention time of thymol in any control or blank samples from the extraction efficiency tests.

Copies of relevant chromatograms are provided for standards of thymol, control cartridge (front segment), and cartridge (front and back segment) spiked with thymol for each ion monitored. Moreover a mass spectrum of thymol is provided to justify the choice of ions monitored.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery was demonstrated. The LOQ, as confirmed by recovery efficiency testing, was 0.5 µg/cartridge which equates to 1.2 µg/m³ based on a flow rate of 1.0 L/minute for a period of 7 hours.

The limit of detection (LOD) was 0.15 µg/cartridge based on 30% LOQ (lowest calibration level) and equates to 0.36 µg/m³.

Accuracy, Retention capacity, Repeatability (precision):

Accuracy and repeatability were assessed at two concentration levels in cartridges and three ions were monitored.

	Thymol m/z 115			Thymol m/z 135			Thymol m/z 150		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n*
0.5	103	-	2	101	-	2	103	-	2
5.0	105	-	2	105	-	2	105	-	2
Overall	104	1.6	4	103	2.7	4	104	1.7	4

Retention capacity was assessed at two concentration levels in cartridges and three ions were monitored.

	Thymol m/z 115			Thymol m/z 135			Thymol m/z 150		
Spike (µg/cartr.)	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n	Mean (%)	RSD (%)	n
0.5	90	2.6	6	94	3.3	6	98	1.8	6
5	100	2.6	6	101	2.5	6	101	2.6	6
Overall	95	6.4	12	97	4.4	12	99	2.7	12

The data indicates that the accuracy and precision of the method are acceptable, since the mean recoveries are well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Extraction efficiency:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with thymol at 5µg/cartridge (10xLOQ) which achieved mean recovery efficiencies from 6 replicate cartridges of 100%, 100% and 99% for thymol ions m/z 115, 135 and 150, respectively.

Cartridge stability:

Satisfactory recoveries were achieved from XAD-2 cartridges spiked with each analyte at 5 µg/cartridge

(10xLOQ) and stored in the dark under ambient conditions for 8 days, which achieved mean recovery efficiencies from three replicate cartridges of 100%, 99% and 100% for thymol ions m/z 115, 135 and 150, respectively.

Stability:

Solvent standards were shown to be stable in solvent solutions stored for 10 and 65 days at nominally 4°C and extract solutions showed no degradation when stored for at least 18 days.

Conclusions

Thymol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored).

Thymol was successfully quantified in air sampling cartridges and the method proposed has been fully validated. Specificity of the method and absence of interference from the test item, or the matrix was demonstrated. The Limit of Quantification was 1.2 µg/m³ for thymol content in air.

The method presented herewith is satisfactory and can be applied to quantify thymol in air via air sampling cartridges.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in air sampling cartridges was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 and was successfully validated.

The method is acceptable for the quantification of thymol in air via air sampling cartridges.

A 2.3.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

KCP 5.2/07 (A 2.3.2.6/01 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in body fluids (plasma and urine) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination of eugenol, geraniol, thymol and methyl-eugenol on plasma and urine from the tested LOQ of 0.01 mg/L up to 0.1 mg/L according to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev.4.</p> <p>The LOQ is 0.01 mg/kg for eugenol, geraniol, thymol and methyl-eugenol for plasma and urine.</p> <p>All mean recovery values for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations ≤ 20%.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/07 (A 2.3.2.6/01 of this dRR)
Report author	Driss F.
Report year	2021e
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body fluid (plasma and urine)
Report No	S20-06626
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17

Deviations from current test guideline None

Previous evaluation No, not previously submitted

GLP/Officially recognised testing facilities Yes, conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: Yes

Principle of the method

In brief, samples of body fluids (plasma and urine) were extracted with acetonitrile. A salt mixture containing magnesium sulphate sodium chloride (urine) or magnesium sulphate, sodium chloride and sodium citrate (plasma) was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) prior to quantification.

Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8
Source and lot/batch no.:	Sigma Aldrich, batch number STBJ4851
Substance content:	99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch:	31 October 2022
Storage conditions:	Ambient

Analysis parameters

Method type GC-MS

Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)

Analytical column: Rtx-1701 (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)

Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)

Injection mode Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz

Purge Flow 50 mL/min at 0.05 min

Injection volume 1 µL (depending on sensitivity)

Injector temperature 250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 15.8 min for thymol

Ionisation mode Electron Impact Ionisation (EI)

Scan type SIM (Selected Ion Monitoring)

Ion source temperature 230°C

Quadrupole temperature 150°C

Solvent delay 6 min

Ions monitored Thymol: 150 (100 ms dwell), 115# (100 ms dwell)

proposed (and/or used) for quantification but all of the ions listed can be used for quantification

Chromatographic conditions for confirmation of thymol

Method type	LC-MS/MS
HPLC system	LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar) LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column	Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure	200 bar
Autosampler temperature	4°C
Column oven temperature	60°C
Injection volume	10 µL (depending on sensitivity)
Mobile phases	Eluent A: Acetonitrile; Eluent B: Water containing 5mM of ammonium acetate

Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0.0	25	75	0.6
	0.5	25	75	0.6
	3.0	95	5.0	0.6
	4.5	95	5.0	0.6
	5.5	25	75	0.6
	7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste

Retention time(s) Approx 3.02 min for Thymol

Mass spectrometric conditions

MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer) SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Negative ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	-4500 V					
Ionspray turbo heater (TEM)	100°C					
Curtain gas (CUR)	Nitrogen set at [25-40] psi	Gas flow 1 (GS1)		Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)		
Collision gas (CAD)	Nitrogen set at 8 psi	Gas flow 2 (GS2)		Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Thymol	149 → 134 (neg)	-85	-10	-20	-15	35

Sample preparation

Urine: An amount of 10 g ± 0.1 g of homogenised urine sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 10 mL acetonitrile was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added

to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

The supernatant was transferred into a polypropylene tube and 2 tubes containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL was transferred a centrifuge tube and 100 µL of toluene is added. The supernatant was evaporated down to 300 µL using a nitrogen evaporator set at 40°C. The volume was adjusted with acetonitrile to 800 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 0.75 µg/mL.

Plasma: An amount of 5 g ± 0.1 g of homogenised plasma sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. The centrifuge tube was capped and vortexed for 5 min. For extraction, exactly 5.0 mL of acetonitrile were added. The centrifuge tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 1 minute. ½ citrate extraction tube containing 4 g of magnesium sulfate, 1 g of sodium chloride was added and the tube was capped, shaken vigorously by hand for 30 seconds and vortexed for 5 minutes. The sample tube was centrifuged for 10 minutes at 4000 rpm at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg PSA and 900 mg magnesium sulfate were added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube was then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 3.0 mL is transferred a centrifuge tube and 50 µL of toluene is added; the supernatant was evaporated to 300 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 400 µL.

The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.075 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 0.75 µg/mL.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/ mL (equivalent to 0.0027 mg/kg to 0.532 mg/kg in both matrices).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effects

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \times C_{\text{Matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
A _{Solv-Std}	Mean peak area of solvent standard
A _{Matrix-Std}	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
C _{Solv-Std}	Nominal concentration of solvent standard in ng/mL
C _{Matrix-Std}	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 70 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C_A	Concentration of analyte in final extract (ng/mL)
A_A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

$R =$	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V_{Ex}	Extraction volume (20 mL)	
V_{End}	Final volume = 1.0 mL	
V_{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000133 for plasma and urine	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ions by GC/MS plus one ion transition by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Thymol		
		<i>m/z</i> 115	<i>m/z</i> 150	<i>m/z</i> 149→134
Plasma	80	(+)5.3	(+)17	(-)12.4
Urine	80	(-)4	(+)25.3	(+)11.2

Matrix effects on the detection of thymol in urine extracts were found to be significant ($\geq 20\%$), but not in plasma extracts. However, as matrix effects on the detection of eugenol and geraniol (see above sections for eugenol and geraniol) in plasma extracts were found to be significant ($\geq 20\%$), matrix-matched standards were used for quantification.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 $\mu\text{g/mL}$, corresponding to analyte concentrations of 0.0027 mg/kg to 0.532 mg/kg in both matrices. Linearity was confirmed for each ion and method.

	Plasma	Urine
<i>m/z</i> 115 (GC-MS)	$y = 183x + 4903$ $r = 0.9989, r^2 = 0.9977$	$y = 145x - 1496$ $r = 0.9993; r^2 = 0.9985$
<i>m/z</i> 150 (GC-MS)	$y = 369x + 2832$ $r = 0.9990, r^2 = 0.9980$	$y = 291x + 879$ $r = 0.9988, r^2 = 0.9975$
<i>m/z</i> 149→134 (LC-MS/MS)	$y = 332x - 2060$ $r = 0.9996, r^2 = 0.9992$	$y = 234x - 1394$ $r = 0.9989; r^2 = 0.9977$

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/L for plasma and urine, for both methods

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 $\mu\text{g/mL}$, equivalent to 0.0027 mg/kg in both matrices.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment <i>m/z</i> 115							
Plasma	0.01	66, 88, 99, 66, 90	82	12	5	91	18
	0.1	84, 93, 96, 123, 102	100	15	5		
Urine	0.01	96, 95, 98, 86, 100	95	6	5	95	6
	0.1	101, 92, 89, 102, 93	95	6	5		
Fragment <i>m/z</i> 150							
Plasma	0.01	82, 94, 107, 72, 103	92	16	5	94	12
	0.1	84, 93, 96, 116, 97	97	12	5		
Urine	0.01	107, 104, 110, 90, 111	104	8	5	100	9
	0.1	98, 91, 89, 104, 92	95	6	5		
Mass Transition <i>m/z</i> 149→134							
Plasma	0.01	105, 74, 95, 75, 92	88	15	5	89	10
	0.1	90, 90, 87, 90, 90	89	2	5		
Urine	0.01	98, 99, 95, 93, 103	98	4	5	95	5
	0.1	98, 88, 86, 92, 96	92	6	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis of samples of matrix-matched standards of thymol, control matrix, and control matrix spiked with thymol. Two fragment ions were monitored by GS/MS and one selected mass transition was monitored by LC-MS/MS.

Additionally, a reagent blank and two control samples per matrix/analyte were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of thymol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of thymol in any investigated matrix plasma and urine, therefore showing that the method is highly specific.

Blank correction was not performed. In addition, two ions were monitored and quantified by GC-MS and one transition was monitored and quantified by LC-MS/MS. The method is specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of thymol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 70 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

Eugenol was found to be stable in final extracts of plasma and urine for 8 and 10 days, respectively, when stored at typically 1°C to 10°C in the dark. However, the mean recovery value for geraniol (see above section for geraniol) in urine re-analysed extracts were out of the range of ± 20 % of the original results. Therefore, samples should be analysed as quickly as possible after extraction.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Thymol - Fragment m/z 115						
Plasma	0	0.01	66, 88, 99, 66, 90	82	18	20
	8		81, 99, 110, 85, 115	98	15	
Urine	0	0.01	111; 91; 94; 90; 103	98	9	6
	10		94; 76; 98; 100*	92	12	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

* Dixon tests was performed to exclude one outlier value

Extraction efficiency:

Not required as part of this study.

Conclusions

Thymol is extracted from plasma and urine matrix using acetonitrile, then quantified by GC-MS using two separate ions and LC-MS/MS using one mass transition for quantification and confirmation of method specificity in plasma and urine.

This analytical method for the determination of thymol content in body fluid matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in both plasma and urine.

The method presented herewith is satisfactory and can be applied to quantify thymol in plasma and urine.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in body fluids (plasma and urine) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and

SANCO/3029/99, rev. 4 and was successfully validated.
The method is acceptable for the quantification of thymol in plasma and urine.

KCP 5.2/08 (A 2.3.2.6/02 of this dRR)

This study has also been submitted within the AIR dossier submitted 28th February 2021 (RMS: Spain).

Comments of zRMS:	<p>The validation of the method for analysis for thymol in body tissue (meat and liver) was not previously evaluated at EU level.</p> <p>The method was successfully validated for the determination eugenol, methyl-eugenol, thymol and geraniol from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4.</p> <p>The LOQ is the lowest validated fortification level for eugenol, methyl-eugenol, thymol and geraniol and was thus successfully established at 0.01 mg/kg in meat and liver for all selected MS fragment ions or mass transitions.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for eugenol, methyl-eugenol, thymol and geraniol are within 70 – 110% with relative standard deviations $\leq 20\%$ and thereby comply with the standard acceptance criteria of the guidance document SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4.</p> <p>The study is acceptable.</p>
-------------------	---

Data point:	CP 5.2/08 (A 2.3.2.6/02 of this dRR)
Report author	Driss F
Report year	2021f
Report title	Validation of the residue method for the determination of eugenol, geraniol, thymol and methyl-eugenol in body tissue (meat and liver)
Report No	S20-06625
Document No	Not applicable
Guidelines followed in study	SANCO/3029/99 rev.4 SANCO/825/00 rev.8.1 ENV/JM/MONO(2007)17
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Principle of the method

Samples of meat and liver were extracted with acetonitrile, if necessary following the addition of a little water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) (for liver only) and aliquots were concentrated prior to quantification. Although the study report refers to thymol, geraniol, eugenol and methyl-eugenol, only thymol is considered in this summary.

Materials and methods

Analytical grade thymol was used as test item.

Test material

Test Standards

Name:	Thymol analytical grade
CAS number:	89-83-8

Source and lot/batch no.: Sigma Aldrich, batch number STBJ4851
Substance content: 99.9% w/w (equivalent to 999 g/kg)
Expiry date of lot/batch: 31 October 2022
Storage conditions: Ambient

Analysis parameters for meat and liver (quantification method)

Method type GC-MS
Instrument: Series 7890A Gas Chromatograph (Agilent Technologies) and 5975C Network mass selective detector (Agilent Technologies)
Analytical column: Rtx-wax (30.0 m x 0.25 mm i.d., 0.25 µm film thickness, Thames Restek)
Carrier Gas Helium (constant pressure / constant flow: 1.1 mL/min)
Injection mode Pulsed splitless (pulse pressure 30 psi/2min), Liner Topaz
Purge Flow 50 mL/min at 0.05 min
Injection volume 1 µL (depending on sensitivity)
Injector temperature 250°C

Column oven temperature programme

Step	Rate [°C/min]	Temperature [°C]	Hold time [min]
1	-	100	2.0
2	5.0	160	0.0
3	15	250	0.0

Retention time: Approx. 15.8 min for Thymol
Ionisation mode Electron Impact Ionisation (EI)
Scan type SIM (Selected Ion Monitoring)
Ion source temperature 230°C
Quadrupole temperature 150°C
Solvent delay 6 min
Ions monitored Thymol for meat: 115# (100 ms dwell), 150 (100 ms dwell), 91 (100 ms dwell)
Thymol for liver: 150 # (100 ms dwell), 115 (100 ms dwell)

proposed (and/or used) for quantification but both of the mass transitions listed can be used for quantification

Analysis parameters for liver (confirmation method)

Method type LC-MS/MS
HPLC system LC20ADXR (pump) + SIL-20ACXR (injector), Shimadzu (HPLC, ≤ 600 bar)
LC30AD Nexera X2 (pump) + SIL-30AC (injector), Shimadzu (UPLC, ≤ 1000 bar)
Column Luna® Phenyl-Hexyl (100 mm x 2.0 mm, 3 µm, 100A, Phenomenex, Art. No. 00D-4256-B0)
Indicative pressure 200 bar
Autosampler temperature 4°C
Column oven temperature 60°C
Injection volume 10 µL (depending on sensitivity)
Eluent A: Acetonitrile;
Mobile phases Eluent B: Water containing 5mM of ammonium acetate

Gradient

Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
0.0	25	75	0.6
0.5	25	75	0.6
3.0	95	5.0	0.6
4.5	95	5.0	0.6
5.5	25	75	0.6
7.5	25	75	0.6

Divert valve 0.0 min to 2.5 min to waste; 2.5 min to 4.0 min to MS; 4.0 min to 7.5 min to waste
Retention time(s) Approx. 3.02 min for Thymol

Mass spectrometric conditions

MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
	SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Negative ion mode for Thymol					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	-4500 V (thymol)					
Ionspray turbo heater (TEM)	100°C					
Curtain gas (CUR)	Nitrogen set at [25-40] psi		Gas flow 1 (GS1)		Nitrogen set at 60 psi (+10 psi if TripleQuad 6500 system used)	
Collision gas (CAD)	Nitrogen set at 8 psi		Gas flow 2 (GS2)		Nitrogen set at 40 psi (+10 psi if TripleQuad 6500 system used)	
Analyte monitored	Mass transition monitored (<i>m/z</i>)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Thymol	149 → 134 (neg)	-85	-10	-20	-15	35

Sample preparation

Meat: An amount of 10 g ± 0.1 g of homogenised meat sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. Exactly 20 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one citrate extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate) was added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The sample tube is then centrifuged at 4000 rpm for 5 minutes at 4°C.

Evaporation:

The supernatant was split into two 8-mL portions and transferred into separate polypropylene tubes. 50 µL of toluene was added to each tube and the supernatant was evaporated to 5 mL under nitrogen at 40°C. the contents of both tubes were combined into a 15 mL polypropylene tube and evaporated to 0.5 mL under nitrogen at 40°C; the volume was adjusted to 1 mL with acetonitrile and the sample was vortexed for 5 minutes. The extract was transferred into a vial and kept at 1°C – 10°C in the dark until injection.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.1 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.0 µg/mL.

Liver: An amount of 10 g ± 0.1 g of homogenised liver sample was weighed into a 50-mL centrifuge tube; fortification was performed at this step when required. 2.5 mL ultra-pure water is added and the tube is capped and vortexed for 5 minutes. Exactly 10 mL acetonitrile is added to the tube which is capped and shaken vigorously by hand for 30 seconds and vortexed for one minute. The contents of one extraction tube (4.0 g of magnesium sulfate, 1.0 g of sodium chloride) was added to the tube which was capped and shaken vigorously by hand for 30 seconds and vortexed for five minutes. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C.

Evaporation:

All the upper acetonitrile phase was transferred in a polypropylene tube and ½ tube containing 150 mg of PSA and 900 mg of magnesium sulfate was added. The centrifuge tube was capped and shaken vigorously by hand for 30 seconds and vortexed for 1 minute. The sample tube is then centrifuged at 4000 rpm for 10 minutes at 4°C. An aliquot of 6.0 mL is transferred a centrifuge tube and 100 µL of toluene is

added; the supernatant was evaporated to 600 µL using a nitrogen evaporator set at 40°C and the volume was adjusted with acetonitrile to 800 µL.

For samples fortified at 0.01 mg/kg, thymol concentration in the final extract was 0.125 µg/mL, and for samples fortified at 0.1 mg/kg, thymol concentration in the final extract was 1.25 µg/mL.

Stock solutions and calibration standards

Thymol stock solution was prepared in acetonitrile. Stock solution concentration was 1000 µg/mL. The stock solution was further diluted to produce a fortification solution which was used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solution and/or preparation of matrix matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of meat which were then fortified with intermediate solvent standard solution.

Calibration standards were in the range 0.02 – 4.00 µg/ mL (equivalent to 0.0025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver).

All solutions were stored at 1°C to 10°C (typically) in a brown glass flask in the dark.

Matrix effects

The effect of matrix meat and liver on the GC-MS and LC-MS/MS response were assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable nominal concentration. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} \times C_{\text{matrix-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Mean peak area of solvent standard
$A_{\text{Matrix-Std}}$	Mean peak area of matrix-matched standard corrected for the mean peak area of the control sample extracts
$C_{\text{Solv-Std}}$	Nominal concentration of solvent standard in ng/mL
$C_{\text{Matrix-Std}}$	Nominal concentration of matrix-matched standard in ng/mL

Accuracy and repeatability

Accuracy was determined by fortification of control samples with known amounts of thymol and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Five recovery determinations were performed at 0.01 mg/kg and at 0.1 mg/kg, respectively. Analysis was performed by single extraction and single injection.

Sample stability

The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 70 days in the dark. After this time freshly prepared dilutions of the stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by triplicate injection. One MS fragment ion per analyte was evaluated.

Extract stability

Following the first analysis, the final extracts of samples fortified at the LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for at least 7 days. After this period, the final extracts were re-analyzed against freshly prepared calibration standards. One MS fragment ion or mass transition per analyte was evaluated.

Calculations

Quantification was performed using calibration plots with a minimum of five different concentration levels covering the required calibration range. Calibration standards were injected spread over the analytical sequence or prior to the analytical sequence with bracketing standards every at least five injections for monitoring of the detector response drift during the run of the sequence. In the case where the calibration standards were injected prior to the analytical sequence the relative deviation of the bracketing standards from the initial linear regression curve was had to be lower than or equal to 25%, otherwise a mathematical correction for the signal drift was applied.

A linear calibration function ($y = a \cdot x + b$) was used to calculate the analyte concentration in final extracts as follows:

$$C_A = \frac{A_A - b}{a}$$

Where:

C _A	Concentration of analyte in final extract (ng/mL)
A _A	Peak area of analyte in the final solution (counts) as obtained by integration with software
a	slope of the calibration curve
b	y-axis intercept of the calibration curve

The residues were then calculated according to the following equations as follows:

R =	$C_A \times EF$	$EF = \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte residue (mg/kg)	
C _A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)	
V _{Ex}	Extraction volume (20 mL)	
V _{End}	Final volume = 1.0 mL	
V _{Ali}	Aliquot of the extract (16 mL)	
W	Sample weight (10 g)	
DF	Dilution factor (for no dilution = 1)	
CF	Conversion factor for ng into µg = 1000	
EF	Extraction Factor = 0.000125 for meat and 0.000133 for liver	

The recovery of a fortification experiment was calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Confirmation of substance identification

Confirmation was achieved by detection and quantification of two ion transitions by LC-MS/MS.

Findings

Matrix effects:

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Thymol (%)		
		m/z 115	m/z 150	m/z 91
Meat	80	66.4	2.1	21.6
Liver	80	m/z 150	m/z 115	m/z 149→134
		6.5	10.7	5.4

Matrix effects were $\geq \pm 20\%$ and deemed to be significant in meat. For liver, it has been shown to be significant for geraniol (See above section for geraniol). Therefore, matrix-matched standards were used for quantification throughout the study.

Linearity:

Linearity was confirmed over the calibration range 0.02 – 4.00 µg/mL, corresponding to analyte concentrations of 0.025 mg/kg to 0.5 mg/kg in meat and 0.0027 mg/kg to 0.532 mg/kg in liver. Linearity was confirmed for each ion and method.

	Meat	Liver
m/z 91 (GC-MS)	y = 281 x + 813 r = 0.9991, r ² = 0.9983	--
m/z 150 (GC-MS)	y = 311 x + 2761 r = 0.9992, r ² = 0.9985	y = 1473 x + 9759 r = 0.9982, r ² = 0.9964
m/z 115 (GC-MS)	y = 209 x + 1528 r = 0.9994, r ² = 0.9988	y = 744 x + 18397 r = 0.9984, r ² = 0.9967
m/z 149→134 (LC-MS/MS)	--	y = 195 x - 386 r = 0.9998; r ² = 0.9997

The method is linear over the calibration range.

LOD, LOQ:

The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for meat and liver, for both methods

The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.02 µg/mL, equivalent to 0.0025 mg/kg in meat and 0.0027 mg/kg in liver.

Accuracy, Repeatability (precision):

Accuracy and repeatability were assessed at two levels of concentrations in meat and liver matrix.

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 91							
Meat	0.1	81, 93, 83, 72, 91	84	10	5	86	11
	0.01	95, 99, 71, 89, 88	88	12	5		
Fragment m/z 150							
Meat	0.01	75, 77, 82, 73, 86	79	7	5	83	11
	0.1	93, 97, 71, 91, 87	88	11	5		
Fragment m/z 115							
Meat	0.01	93, 100, 96, 79, 103	94	10	5	91	11
	0.1	94, 99, 71, 91, 88	89	12	5		

Thymol							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Fragment m/z 150							
Liver	0.01	87; 87; 74; 85; 77	82	7	5	91	12
	0.1	104; 95; 100; 100; 104	101	4	5		
Fragment m/z 115							
Liver	0.01	97; 91; 73; 82; 82	85	11	5	93	11
	0.1	103; 97; 103; 98; 103	101	3	5		
Transition m/z 149→134							
Liver	0.01	67; 74; 81; 79; 90	78	11	5	83	9
	0.1	81; 89; 91; 85; 88	87	4	5		

No observable peak was detected in any control sample extract; Recoveries are without any blank correction.

The data indicates that the accuracy and precision of the method is acceptable, in that the mean recoveries is well within the range (70 -120%) and the RSD obtained is well below the limit value of 20%.

Specificity:

Specificity was studied by analysis samples of matrix-standards of thymol, control matrix, and control matrix spiked with thymol. Two fragment ions were monitored by GS/MS and one selected mass transition was monitored by LC-MS/MS.

Additionally, a reagent blank and two control samples per matrices/analytes were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of thymol. For all fragment ions or mass transition, the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes in any investigated matrix meat and liver, therefore showing that the method is highly specific.

Copies of relevant chromatograms are provided for matrix-matched standards, reagent blank, control matrix and fortified matrix at both fortification level for each ion monitored. Moreover, mass spectra of geraniol are provided to justify the choice of ion transition monitored.

Stability of standards and extracts:

Stock stability:

The mean peak areas of the stored diluted stock solutions were within ± 10 % of the mean peak areas of the freshly prepared diluted stock solutions, indicating that stock solutions are stable when stored at 1°C to 10°C for 70 days in the dark, which was sufficient to cover the length of time they were used in this study.

Stability of sample extracts:

The mean recovery values for thymol re-analysed extracts were in the range 70-110% and $\pm 20\%$ of the original result for liver but not for meat. For liver, as the acceptance criteria were not fulfilled for geraniol (see above and above section for geraniol), final extracts of meat and liver are considered to be unstable for 17 and 8 days, respectively when stored at typically 1°C to 10°C in the dark. Therefore, extracts should be analysed within 24 hours in order to prevent any degradation.

The results obtained are summarised in the table below.

Matrix	Interval days of storage* (1 st to 2 nd Injection)	Fortification level (mg/kg)	Recovery (%)	Mean recovery (n = 5) (%)	Rel. Std. Dev. (n = 5) (%)	Difference (in %) of recoveries after storage to recoveries before storage in % ^a
Thymol - Fragment <i>m/z</i> 150						
Meat	0	0.01	75; 77; 82; 73; 86	79	7	(+)30
	17		108; 99; 108; 92; 107	103	7	
Liver	0	0.01	87; 87; 74; 85; 77	82	7	(+)18
	8		100; 98; 83; 104; 98	97	8	

* Time interval between 1st and 2nd injection.

^a Difference = ((Mean of 2nd injection – Mean of 1st injection)/ Mean of 1st injection) * 100

Extraction efficiency:

Not required as part of this study.

Conclusions

Thymol is extracted from meat or liver matrix using acetonitrile, then quantified by GC-MS using three separate ions for meat samples and quantified by GC-MS using two separate ions and by LC-MS/MS following one ion transition for liver samples. The method is specific.

This analytical method for the determination of thymol content in tissue matrix has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for thymol in both meat and liver.

The method presented herewith is satisfactory and can be applied to quantify thymol in meat and liver.

Assessment and conclusion by applicant:

The validation of the method for analysis for thymol in body tissue (meat and liver) was not previously evaluated at EU level. It was performed under GLP according to Guidelines SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 and was successfully validated.

The method is acceptable for the quantification of thymol in meat and liver.

A 2.3.2.7 Other Studies/ Information

No new or additional studies have been submitted.