REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: Cymoxanil 33% + Zoxamide 33% WG

Product name(s): **Lieto 66 WG**

Chemical active substance(s):

Cymoxanil, 330 g/kg

Zoxamide, 330 g/kg

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(product re-registration)

Applicant: Sipcam Oxon S.p.A.

Submission date: 30/12/2020

MS Finalisation date: September 2021

Revision date: December 2021

DATA PROTECTION CLAIM

Under Article 59 of Regulation 1107/2009/EC, the applicant claims data protection for these studies. The data protection status and corresponding justification as valid for the respective country will be confirmed in the respective PART A.

STATEMENT FOR OWNERSHIP

The summaries and evaluations contained in this review report may be based on unpublished proprietary data submitted for the purpose of the assessment undertaken by the regulatory authority that prepared it. Other registration authorities should not grant, amend, or renew a registration on the basis of the summaries and evaluation of unpublished proprietary data contained in this review report unless they have received the data on which the summaries and evaluation are based, either –

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- following expiry of any period of exclusive use, by offering – in certain jurisdictions – mandatory compensation, unless the period of protection of the proprietary data concerned has expired.

Version history

When	What
30th December 2020	Submission of initial Version 0 by the applicant. *
21st April 2021	Version revised by the applicant, highlighting studies already evaluated during product authorisation and confirmatory-like studies which are under evaluation by the RMS for Zoxamide in an interzonal procedure.
September 2021	Evaluated after renewal of zoxamide
December 2021	Revised version, addressing the comments of MSs.

* This version ~~0~~ 5 has been corrected and provided as corrected version to the zRMS due to several bugs in the MACRO. Therefore, the "Table of Contents" and the automatic numberings and headings are now functioning again in this document.

Some missing information in the overview table (Table 5.2-2: Validated methods for the generation of pre-authorisation data - zoxamide) and on two study summaries (Extraction efficiency) have been added. The new/changed information is highlighted in green.

Please note: since the applicant used a grey to highlight unchanged data from the previous version, the evaluator of residue analytical methods except commenting grey boxes used a blue to highlight his own current text.

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5 Analytical methods

This document summarises the analytical methods on the plant protection product Cymoxanil 33% + Zoxamide 33 % WG (trade name e.g. Reboot), a WG formulation containing 330 g/kg zoxamide and 330 g/kg cymoxanil, for re-registration in EU countries. Cymoxanil 33% + Zoxamide 33 % WG is a product on the EU market. It is a fungicide that has been jointly developed by the companies Gowan Crop Protection Ltd. (legal successor of the company Gowan Comercio Internacional e Servicos Limitada) and Sipcam Oxon S.p.A. (legal successor of the company OXON Italia S.p.A.). Cymoxanil 33% + Zoxamide 33 % WG is a fungicide, for which re-registration according to article 43 of regulation 1107/2009 is requested on behalf of Gowan Crop Protection Ltd., UK. The dossier follows the data requirements of

- Regulation (EC) No. 544/2011 for the active substance cymoxanil,
- Regulation (EC) No. 283/2013 for the active substance zoxamide and
- Regulation (EC) No. 284/2013 for the plant protection product Cymoxanil 33% + Zoxamide 33 % WG.

This document is for the renewal of the authorisation of the product according to Article 43 of Regulation (EC) No 1107/2009, following the renewal of approval of the active substance zoxamide according to Regulation (EU) 2018/1981 of 13 December 2018.
The aim of this step of the art. 43 process is to update the existing dossier information with regard to and limited to the information on the active substance zoxamide as follows:

- To comply with data requirements or criteria which were not in force when the authorisation of the plant protection product was granted and
- to demonstrate that the product meets the requirements set out in the Regulation on the renewal of the approval of the active substance zoxamide to comply with provisions of article 29 of Regulation (EU) No 1107/2009.

This dossier contains the consolidated version of the previous assessment for the parts which do not require a re-evaluation, including all assessments and data on cymoxanil.

The document is based on the Registration Report provided by Spanish INIA in January 2013 and UK CRD in October 2014 and inhibits the evaluation results of the zRMS UK for product approval in the central EU zone. Unchanged data from the previous version are highlighted in grey.

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are available for the active substance(s) and relevant impurities in the plant protection product.
Noticed data gaps are:

None.

Cymoxanil data are not under evaluation in this submission.

For zoxamide, sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definition for risk assessment (see EFSA, 2017).

Please note: Sufficiently sensitive and selective analytical methods are available for monitoring/enforcement of zoxamide in plants and animal commodities according to Reg. (EU) 2017/171 and as requested by EFSA (2017).
In the context of this re-registration request noticed data gaps are:

None.

For the active substance **zoxamide** the company Gowan Crop Protection Ltd. as sole notifier submitted in 2014 a dossier to Latvia as RMS and France as co-RMS to support the renewal of approval of the active substance zoxamide on EU level.

zRMS agrees with the approach of the applicant. The data submitted are sufficient to re-authorize the product. However, since new residue definitions for risk assessment in plants were proposed (2017, EFSA) part of the submitted data within this consolidated dossier is therefore still under evaluation by the RMS Latvia.

Please note that data requested by EFSA (2017) and in the EC Renewal Report (2017) to conclude on the residue definitions of zoxamide in plant and animal commodities as well as environmental compartments are currently under evaluation in an ongoing interzonal procedure with Latvia as the RMS for zoxamide and inter-zonal RMS for this procedure, together with the relevant countries as cMSs. For final residue definitions for monitoring/enforcement together with the underlying data and methods, it is therefore referred to this procedure.

It will be followed by the outstanding review of the maximum residue levels (MRLs) under Article 12 of Regulation (EC) No 396/2005.

~~For zoxamide, sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.~~
The section B5 conclusion of the applicant included in the national part A, as acceptable, the evaluator pasted also here:

Commodity/crop	**Supported/ Not supported**
Plant: grape, potato, tomato, cucumber and onion raw agricultural and processed comodities	Supported
Animals not relevant	
Soil	Supported
Water	Supported
Air	Supported
Body fluids and tissues	Supported

Cymoxanil data are not under evaluation in this submission.

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)

An overview on the acceptable methods for analysis of the active substances in the plant protection product Cymoxanil 33% + Zoxamide 33 % WG (trade name e.g. Reboot; synonym developmental names e.g. Harpon XF – 98083, SIP 40936; WG formulation containing 330 g/kg zoxamide and 330 g/kg cymoxanil) is provided as follows.

A HPLC-UV method for the determination of zoxamide and cymoxanil in the presence of each other and the co-formulants of the WG formulation Cymoxanil 33% + Zoxamide 33 % WG has been developed by Diogo (2003; report no. DAS-AM-02-051). The method complies with SANCO/3030/99 rev. 4 (2000).

An additional method is available from De Ryckel (2019; report no. 24718), which complies with SANCO/3030/99 rev. 5 (2019). With this method, it is also possible to check the ratio of the enantiomers of the racemate zoxamide.

Comments of zRMS:	The study is still accepted.

Reference:	**KCP 5.1.1/01**
Report	Diogo, A., 2003: Analytical method and validation for the determination of cymoxanil and zoxamide in Harpon XF – 98083 formulation Dow AgroSciences Industrial Ltda., Brazil, Report no. DAS–AM–02–051, GLP, Not published
Guideline(s):	U.S. EPA OPPTS Test Guideline 830.1800
Deviations:	No
Acceptability:	Yes

Materials and methods

A liquid chromatographic method was validated for the analytical determination of cymoxanil and zoxamide in Harpon XF 98083 formulation. Quantification was performed by HPLC-UV, using the internal standard benzophenone dissolved in acetonitrile (0.5 g/L). Samples were prepared by weighing 240 mg of the formulation and adding 100 ml of standard solution.

HPLC parameters	
Column	Partisil 10 ODS Whatman- 4.6 x 250 mm x 10 µm, Part # 4223-001 lot OTCCBB20
Column flow rate	3 mL/min
Detector wavelength	UV, 240 nm
Eluent	64.7% (v/v) water – 35% (v/v) acetonitrile – 0.3% (v/v) phosphoric acid
Retention time	Cymoxanil: ~2 min. Benzophenone: ~6 min. Zoxamide: ~13 min.

The parameters specificity, linearity, accuracy and precision were checked. Typical calibration curves and chromatograms are presented in the report.

Specificity

No significant interferences were detected by comparing retention times of active substances after blank injection, internal standard injection, solvent injection and standard injections.

An impurity in the zoxamide technical material was found to co-elute with the internal standard. No area data or calculations relating to this impurity were provided in the report; however, it is stated that the impurity represented only about 0.8% of the total peak area and so is not expected to significantly affect the results. This is accepted.

Whilst minor components in the formulation blank were not totally resolved from cymoxanil, it appears as though these interferences did not contribute to the peak area for cymoxanil and therefore did not affect the results.

Linearity

Nominal concentration of both cymoxanil and zoxamide in prepared samples of the product = (0.33 x 240) / 100 = 0.792 mg/ml.

Single determinations were made at seven concentrations and a linear relationship between peak area and concentration was noted from 0.27 to 1.36 mg/mL (34-172% nominal concentration) for Cymoxanil (r^2 = 0.9995) and from 0.40 to 2.02 mg/ml (51-255% nominal concentration) for zoxamide (r^2 = 0.9999).

- Cymoxanil Y= 8089284X + 226256; r2 = 0.9995
- Zoxamide Y= 5718997X + 94055; r2 = 0.9999

Accuracy

Eight samples for analysis were prepared by dissolving cymoxanil analytical standard, zoxamide analytical standard and the formulation blank in 100 ml of the internal standard solution.

Cymoxanil:

Recovery data were obtained over the active content range 21.66% to 42.75% (w/w) with an average recovery of 99.9% over eight samples.

Zoxamide:

Recovery data were obtained over the active content range 20.44% to 41.78 % (w/w) with an average recovery of 100% over eight samples.

The relative standard deviation was ±0.37% at an average concentration of 33.52% for cymoxanil and ±0.53% at an average concentration of 33.39 for zoxamide.

Method precision (repeatability)

Five samples were prepared and analysed on two separate days. The analyses produced a relative standard deviation of 0.37 % for cymoxanil and 0.53% for zoxamide at an average concentration of 33.89% and 33.68%, respectively.

The % RSD results were acceptable based on the Horwitz equation.

Solution stability

The solution stability was determined by analysing a sample solution one week after the initial analysis. The results obtained for the aged sample were found to be equivalent to the original results.

EVALUATION, SUMMARY AND CONCLUSION BY REGULATORY AUTHORITY
Name of authority: Chemicals Regulation Directorate, UK

Reviewer's comments:
Method DAS–AM–02–051 is suitably validated for the quantification of Cymoxanil and zoxamide in preparations of 'Cymoxanil 33% + Zoxamide 33% WG' as per SANCO /3030/99 rev. 4.

Comments of zRMS:	That study meets the Sanco/3030/99 rev.5 requirements and can be applied for analysing active substances in the PPP.

Evaluation results of Spanish INIA:

Study Comments: IIIA 5.2.2/01 & 02	**IIIA 5.2.2/01** HPLC/UV method **DAS-AM-02-051** has been validated for the plant protection product HARPON XF 98083 formulation using benzophenone as internal standard and monitoring λ= 240 nm for quantification the active substances Cymoxanil and zoxamide with good results. All spectra are clear and show the purity of both active substances in the plant protection product. Chromatograms have been demonstrated the specificity of the method for both cymoxanil and zoxamide. None interferences are close to the signal peaks, and just small one is on retention time from internal standard but doesn't represent more than 0.8 %. Linearity is demonstrated in the limits proposed (0.27 mg/mL to 1.36 mg/mL for Cymoxanil, which is equivalent to 0.34 to 1.7 times the nominal concentration and from 0.40 mg/mL to 2.02 mg/mL for zoxamide, which is equivalent to 0.51 to 2.6 times the nominal concentration); However, concentration data of the linearity range should refer to both the mass fraction in the original sample (mg/Kg) and to the concentration in the extract (mg/mL). Precision have been demonstrated analysing five times a nominal sample and checking the RSD results with Horwitz equation; furthermore, recovery experiments corroborate the accuracy of the method. Roughness of the method has been shown by changing the eluting settings and affording same selectivity at different retention times. Cymoxanil is eluted as one signal peak for the (E/Z) isomers. According to EFSA conclusions the ratio of E/Z isomers must be specified in cymoxanil. Although this point was left open the method of analysis should be capable to separate the isomers. On the other hand, zoxamide is optically active and the separation of the optic isomers could be desirable in the chromatogram traces, however, it is not an essential requirement yet. **IIIA 5.2.2/02** Applicability of the method **DAS-AM-02-051** has been shown, affording good results with "real" samples.
Agreed endpoint: IIIA 5.2.2/01 & 02	**IIIA 5.2.2/01** The method **DAS-AM-02-051** is suitable for the determination of cymoxanil and zoxamide in Harpon XF 98083 formulation.

Comments of zRMS:	Accepted. The study meets the Sanco/3030/99 rev.5 requirements

Reference:	**5.2.1.1/01 (KCP 5.1.1/02)**
Report	De Ryckel, B., 2019: Validation of analytical method of cymoxanil and zoxamide content and physico-chemical properties and storage stability of Cymoxanil 33% + Zoxamide 33% WG Gowan Crop Protection Limited, UK Walloon Agricultural Research Centre (CRA-W), Belgium, Report No. 24718, GLP, Not published

Guideline(s):	SANCO/3030/99 rev. 4 (2000) OECD 204 (2014) SANCO/3030/99 rev. 5 (2019)
Deviations:	No
Acceptability:	Yes

Materials and methods

An HPLC-DAD method has been validated for the determination of cymoxanil and zoxamide content (with ratio of the zoxamide isomers) in the presence of each other and of the blank formulation from Cymoxanil 33% + Zoxamide 33% WG, a water dispersible granule (WG) formulation containing 33% of cymoxanil and 33% of zoxamide (nominal concentrations). The method **MET-24718** is based on the MET-24604, which has been developed to determine the zoxamide content, and has been adapted to additionally allow determining the cymoxanil content. It has been validated according to SANCO/3030/99 rev. 4 and rev. 5 and OECD 204 (2014). The cymoxanil content, the zoxamide content and the ratio of its isomers were measured after dispersion of the test item Cymoxanil 33% + Zoxamide 33% WG and dissolution of active substances in acetonitrile and dilution in ethanol. The separation was achieved using reverse phase liquid chromatography, followed by UV detection and external standard. Typical retention times (see on pages 50 to 55) and UV spectra (see on pages 59 and 60) are included in the report. The identity of cymoxanil and of zoxamide isomers was confirmed by LC-MS/MS.

For test item analysis, weigh (to the nearest 0.1 mg) about 264 mg of test item into a 100 mL volumetric flask. Add acetonitrile and disperse the test item during minimum 10 minutes. Fill to the mark (at 20°C ±1°C) with acetonitrile. Mix thoroughly and filter (0.45 µm PTFE filter). Transfer 5 mL of this solution into a 25 mL volumetric flask and dilute to the mark with ethanol. This solution is prepared in triplicate. Mix well and measure via HPLC-DAD.

Calibration solutions were prepared with a.s. standards (the R- and S-zoxamide and cymoxanil): Weigh (to the nearest 0.1 mg) about 10 mg of zoxamide R-isomer analytical standard, 10 mg of zoxamide S-isomer analytical standard and 20 mg of cymoxanil analytical standard into a 25 mL volumetric flask. Add acetonitrile and place the flask in an ultrasonic bath until complete dissolution (minimum during 10 minutes). Fill to the mark at 20°C ± 1°C with acetonitrile. Mix thoroughly before injection. In this manner, six calibration solutions with different active substance concentrations were prepared to establish a calibration curve.

Blank formulation was available for spiking experiments / determination of recoveries.

Equipment:

Instrument:	Alliance - Waters 2695 Separation Module
Column:	Phenomenex Lux Cellulose-3, 3 µm, 250 x 4.6 mm i.d
Detector	Waters 996 Photodiode Array Detector

Mobile phase:	A: water + 0.1% TFA B: methanol + 0.1% TFA

Time (minutes)	% A	% B
0.0	30	70
2.0	30	70
6.0	20	80
13.0	20	80
13.1	30	70
15.0	30	70

Flow rate:	1 mL/min
Column temp.:	40°C
Injection volume:	10 µL
Retention time:	Cymoxanil: ± 4.6 minutes R-isomer of zoxamide: ± 10.6 minutes S-isomer of zoxamide: ± 13.2 minutes
Run time:	15 min
Ion mode	Zoxamide: 336.6 → 187.1 (quantification) 336.6 → 159.0 (confirmation) Cymoxanil: 198.2 → 128.0 (quantification) 198.2 → 111.0 (confirmation)

It is recommended after the injection sequence to clean the column with 100% methanol for at least 2 hours to eliminate interfering substances accumulating on the HPLC column.

Analyte identity has been confirmed by LC-MS/MS:

Equipment:

Instrument:	Acquity UPLC Waters HPLC
Column:	Phenomenex Lux Cellulose-3, 3 µm, 250 x 4.6 mm i.d
Detector	Tandem Quadripole Mass Spectrometer (TQD) Waters
Mobile phase:	A: water Ultra pure + 0.1% NH_4OH 5N B: acetonitrile, U-LC reagent grade

Time (minutes)	% A	% B
0.0	40	60
0.5	40	60
10.5	25	75
10.51	5	95
12.0	5	95
12.01	40	60
13.00	40	60

Flow rate: Column temp.:	0.8 mL/min RT
Sample temp.:	4°C
Injection volume:	1 µL (partial loop)
MS/MS conditions:	Ionisation : positive electrospray (ESI +) Capillary voltage : 1.5 kV

	Cone gas flow : 50 L/Hr (nitrogen) Desolvation gaz flow : 700 L/Hr (nitrogen) Collision gaz flow : 0.30 mL/min (argon) Source temperature : 140°C Desolvation temperature : 350°C Dwell time : 0.05 sec **Zoxamide (MW 336.6):** Parent ion = 335.02 amu Cone voltage : 38 v Quantification on daugther ion m/z = 187.10 amu Collision energy : 25 v Confirmation on daughter ion m/z = 159.00 amu Collision energy : 38 v **Cymoxanil (MW 198.2):** Parent ion = 199.00 amu Cone voltage : 23 v Quantification on daugther ion m/z = 128.0 amu Collision energy : 8 v Confirmation on daughter ion m/z = 111.0 amu Collision energy : 18 v
Run time:	13 min
Retention time:	Cymoxanil: ± 4.1 minutes R-isomer of zoxamide: ± 7.2 minutes S-isomer of zoxamide: ± 8.7 minutes

Validation - results and discussions

Table 5.2-1: Methods suitable for the determination of zoxamide (R, S and sum) and cymoxanil in Cymoxanil 33% + Zoxamide 33% WG

	Cymoxanil	R-isomer of zoxamide	S-isomer of zoxamide	Zoxamide sum of isomers
Author(s), year	De Ryckel B. (2019), Report No. 24718			
Principle of method	HPLC-DAD method			
Linearity	Curve 1 (first weighing - 3 dilutions) The response of cymoxanil is linear in the range 26.6–106.2 µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 2 (second weighing - 3 dilutions) The response of cymoxanil is linear in the range 50.9–203.5µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 3 (2 x 3 dilutions) The response of cymoxanil is linear in the range 26.6–203.5 µg/mL with r^2 = 0.9996 [r = 0.9998]. Individual calibration data and calibration line equation presented in the study report.	Curve 1 (first weighing - 3 dilutions) The response of *R*-isomer of zoxamide is linear in the range 15.2 – 61.0 µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 2 (second weighing - 3 dilutions) The response of *R*-isomer of zoxamide is linear in the range 24.9 – 99.5 µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 3 (2 x 3 dilutions) The response of *R*-isomer of zoxamide is linear in the range 15.2 – 99.5 µg/mL with r^2 = 1.0000 [r = 1.0000]. Individual calibration data and calibration line equation presented in the study report.	Curve 1 (first weighing - 3 dilutions) The response of *S*-isomer of zoxamide is linear in the range 20.7 – 83.0 µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 2 (second weighing - 3 dilutions) The response of *S*-isomer of zoxamide is linear in the range 25.4 – 101.7µg/mL with r^2 = 1.0000 [r = 1.0000]. Curve 3 (2 x 3 dilutions) The response of *S*-isomer of zoxamide is linear in the range 20.7 – 101.7µg/mL with r^2 = 1.0000 [r = 1.0000]. Individual calibration data and calibration line equation presented in the study report.	See linearity of both isomers
Range of method	5.03 – 38.53 % w/w	2.89 – 18.85 % w/w	3.93 – 19.26 % w/w	6.82 – 38.11 % w/w
Precision / repeatability mean %, n = 6 (%RSD)	Mean : 32.75 % w/w RSD : 0.15% Horrat (Hr) : 0.28 *	Mean : 16.67 % w/w RSD : 0.57 % Horrat (Hr) : 0.32 *	Mean : 16.24 % w/w RSD : 0.47 % Horrat (Hr) : 0.27	Mean : 32.91 % w/w RSD : 0.49 % Horrat (Hr) : 0.31
Accuracy **mean % recovery values** **% RSD values** **n = 6--**	Level : 25.41 % w/w (n = 2): 99% (80% of nominal concentration) Level : 32.59 % w/w (n = 2): 98.2% (100% of nominal concentration)	Level : 13.60 % w/w (n = 2): 100.7 % (80% of nominal concentration) Level : 16.81 % w/w (n = 2): 100.9	Level : 13.60 % w/w (n = 2): 100.7 % (80% of nominal concentration) Level : 16.81 % w/w (n = 2): 100.8	Level : 26.86 % w/w (n = 2): 100.7 % (80% of nominal concentration) Level : 33.19 % w/w (n = 2): 100.8

	Level : 38.65 % w/w (n = 2): 101% (120% of nominal concentration) Overall mean: 99.4 %, RSD: 1.41%	% (100% of nominal concentration) Level : 20.55 % w/w (n = 2): 99.2 % (120% of nominal concentration) Overall mean: 100.3 %, RSD: 1.30%	% (100% of nominal concentration) Level : 20.55 % w/w (n = 2): 100.2 % (120% of nominal concentration) Overall mean: 100.5 %, RSD: 0.66%	% (100% of nominal concentration) Level : 40.57 % w/w (n = 2): 99.7 % (120% of nominal concentration) Mean (n = 6) Overall mean: 100.4 %, RSD: 0.97%
Interference/ Specificity	Specific; Interference < 3%	Specific; Interference < 3%	Specific; Interference < 3%	Specific; Interference < 3%
Confirmation	Identity confirmed via LC-MS/MS	Identity confirmed via LC-MS/MS	Identity confirmed via LC-MS/MS	Identity confirmed via LC-MS/MS
Analysis of technical item	--	Mean : 49.44 % w/w RSD : 0.65% Horrat (Hr) : 0.43 **	Mean : 48.17 % w/w RSD : 1.02% Horrat (Hr) : 0.69 ***	Mean : 97.62 % w/w RSD : 0.83% Horrat (Hr) : 0.62

Conclusion

The HPLC-DAD method has been validated for the determination of cymoxanil and zoxamide content (with ratio of the zoxamide isomers) in the presence of each other and of the blank formulation from Cymoxanil 33% + Zoxamide 33% WG, a water dispersible granule (WG) formulation containing 33% of cymoxanil and 33% of zoxamide (nominal concentrations). The method MET-24718 is based on the MET-24604, which has been developed to determine the zoxamide content, and has been adapted to additionally allow determining the cymoxanil content. It has been validated according to SANCO/3030/99 rev. 4 and rev. 5 and OECD 204 (2014).

(De Ryckel B. 2019)

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

Zoxamide

No relevant impurities. Please refer to dRR Part C (confidential information) for further details.

Cymoxanil

No relevant impurities. Please refer to dRR Part C (confidential information) for further details.

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

With respect to toxicological, eco-toxicological or environmental aspects the product does not contain-any relevant formulants. Therefore, a special analytical method and validation is not needed.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are no CIPAC methods available for zoxamide and cymoxanil.
CIPAC method 419/WG/M/3 is available for the determination of Cymoxanil active substance in Cymoxanil water-dispersible granules.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

Zoxamide

For zoxamide, reference is made to the available analytical methods evaluated at EU level (please refer to the RAR, 2017). Additional methods were developed and validated, as needed to support studies in the different scientific areas. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

An overview on the acceptable methods for analysis of residues of zoxamide for the generation of pre-authorisation data is given in the following table.

Table 5.2-2: Validated methods for the generation of pre-authorisation data – zoxamide

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: Zoxamide				
Grape fruits Grape juice Raisins	Primary	0.01 mg/kg	GC-ECD	Burdge *et al.*, 1998 Report no. 34-98-150 DPDB 81828 EU agreed method (RAR, 2017)
	Confirmatory		GC-MS	
	Radiovalidation		--	
	ILV			Szuter, 1998 Report no. 34-98-177 DPDB 81832 EU agreed method (RAR, 2017)
Component(s) analysed: Zoxamide				
Grape fruits Grape juice Wine Raisins Potato tubers Potato flakes Potato chips	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 Report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 Report no. P3114G EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G10186-01-VMPL
	ILV			Schlewitz, 2014 Report no. R B4023 EU agreed method (RAR 2017)
Component(s) analysed: Zoxamide				
Grape fruits Potato tubers	Primary Confirmatory	Grapes: 0.01 mg/kg Potatoes: 0.005 mg/kg	LC-MS/MS (QuEChERS)	Luciani, 2010 Report no. AGRI 012/10 GLP DEC EU agreed method (RAR 2017)
Component(s) analysed: Zoxamide				
Grape fruits	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Luciani, 2010 Report no. AGRI 010/10 GLP DEC EU agreed method (RAR 2017)
Component(s) analysed: Zoxamide				
Grape fruits	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Luciani, 2016 Report no. AGRI 009/15 GLP HAR
Component(s) analysed: Zoxamide				
Grape fruits Must Young wine Bottled wine	Primary	0.01 mg/kg	GC-ECD	Romanini, 2011 Report no. CREG2117 Report no. CREG2120

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: Zoxamide				
Grape fruits Must Wine	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Perboni, 2017 Report no. RAU-049-15
Component(s) analysed: Zoxamide				
Tomato fruits Purre Paste	Primary	0.01 mg/kg	GC-ECD	Burdge *et al.*, 1999 Report no. 34-99-111 Applied in CEMS-2967
	Confirmatory		GC-MS	
	Radiovalidation		--	
	ILV			Bruns, G., Gottschalk, R., 1999 Report no. 34-99-188
Component(s) analysed: Zoxamide				
Tomato fruits Juice Purre Canned tomatoes	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Tetuan, 2016 Report no. 15 F CL GW P/A
Component(s) analysed: Zoxamide				
Tomato fruits Juice Puree Canned	Primary	0.01 mg/kg	GC-ECD	Romanini, 2011 Report no. CREG2118
Component(s) analysed: Zoxamide				
Potato tubers flakes chips	Primary Confirmatory	0.01 mg/kg	GC-MS/MS LC-MS/MS (QuEChERS)	Tetuan, 2011 Report no. 10 F PT GW P/A Report no. 10 F PT GW P/B
Component(s) analysed: Zoxamide				
Lettuce	Primary Confirmatory Method AGRI BPL 040	0.01 mg/kg	LC-MS/MS	Luciani, 2016 Report no. AGRI 013/12 Report no. AGRI 014/12
Component(s) analysed: Zoxamide				
Tomato fruits	Primary Confirmatory	0.02 mg/kg	LC-MS/MS (QuEChERS)	Tetuan, 2016 Report no. 15 F CL GW P/A
Component(s) analysed: Zoxamide				
Grape fruits Must Pomace Wine	Primary	0.01 mg/kg	GC-ECD	Wais, 2001 report no. 734580 EU agreed method (RAR 2017)

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: R- and S-zoxamide and sum				
Potato tubers Potato flakes Potato chips Pickled silverskin Onion bulbs	Primary / ILV Confirmatory	0.005 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G10186-01-VMPL
Component(s) analysed: R- and S-zoxamide and sum				
Grape fruits Grape juice Wine Raisins Tomato fruits Canned tomatoes Potato tubers Potato flakes Fried potatoes Cucumber fruits Onion bulbs	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 Applied in several BPL residue trials up from 2018.
Component(s) analysed: Zoxamide and its metabolites RH-141455 and RH-141452				
Potato tubers Potato flakes Potato chips	Primary	0.02 mg/kg	GC-ECD	Meyer *et al*., 1998 Report no. 34-98-142 DPDB 81812 EU agreed method (RAR 2017)
	Confirmatory		GC-MS	
	Radiovalidation		--	
	ILV			Bruns & Nelson, 1998 Report no. 34-98-180 DPDB 81816 EU agreed method (RAR, 2017)
Component(s) analysed: Zoxamide and its metabolites RH-141455 and RH-141452				
Potato tubers Potato flakes Fried potatoes Onion bulbs	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 Applied in several BPL residue trials up from 2018.
Component(s) analysed: Zoxamide and its metabolite RH-141452				
Grape berries Grape juice Wine Raisins Cucumber fruit Tomato fruit Canned tomatoes	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 Applied in several BPL residue trials up from 2018.

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: RH-141455 and RH-141452				
Potato tubers Potato flakes Potato chips	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS (QuEChERS)	Weber & Giesau, 2013 Report no. S12-03951 EU agreed method (RAR 2017)
Pickled silverskin onions	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G10186-01-VMPL
Component(s) analysed: Zoxamide, RH-150721				
Wine	Primary	0.01 mg/kg each	GC-ECD (Rtx-5 column)	Burdge *et al*., 1998 Report no. 34-98-179 DPDB 81841 EU agreed method (RAR, 2017)
	Confirmatory		GC-ECD (HP-35 column)	
Component(s) analysed: RH-150721				
Grape fruits Grape juice Wine	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber & Giesau, 2013 Report no. S12-03950 EU agreed method (RAR, 2017)
Component(s) analysed: Zoxamide and its metabolites RH-141452, RH-24549, RH-129151, RH-50721, RH-141288				
Grape juice Wine Raisins Canned tomatoes	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 Applied in several BPL residue trials up from 2018.
Component(s) analysed: RH-150721, RH-141452, RH-141455				
Grape fruits Grape juice Wine Raisins Potato tubers	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber *et al*., 2016 Report no. S12-03952 EU agreed method (RAR, 2017)
Component(s) analysed: RH-141455				
Blood plasma	Primary Confirmatory	0.104 µg/mL	LC-MS/MS	xxx 2019 Report no. U-19044 xxx, 2020 Report no. U-19102 Report no. U-19071
Component(s) analysed: RH-150721				
Rat feed	Primary	0.091 mg/mL	HPLC-UV	xxx, 2020 Report no. U-19162

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: RH-141455				
Rat feed	Primary	0.005 mg/mL	HPLC-UV	xxx, 2019 Report no. U-19069
Component(s) analysed: Zoxamide				
Honey bee royal jelly	Primary Confirmatory	0.5 µg/g	HPLC-MS/MS	Picard, 2018 Report no. 12791.6307
Component(s) analysed: Zoxamide				
Honey bee royal jelly	Primary	46.40 mg/L	HPLC-UV	Ruhland, 2018 Report no. 17 48 BAC 0005
Aqueous sugar solution with royal jelly	Primary	8.594 mg/L	HPLC-UV	Scheller, 2020 Report no. 17 48 BLC 0005
Pollen, nectar, flowers, bees	Primary Confirmatory	0.005 mg/L	LC-MS/MS	Schnurr, 2020 Report no. 18 48 BFB 0001 Report no. 19 48 BFB 0001
Component(s) analysed: Zoxamide				
Soil	Primary Confirmatory	0.01 mg/kg	GC-ECD GC-MS	Guo *et al.*, 1996 Report no. 34-96-91 DPDB 81847 EU agreed method (RAR, 2017)
Soil	Primary	0.01 mg/kg	GC-ECD	Guo *et al.*, 1998 Report no. 34-98-126 DPDB 81851 EU agreed method (RAR, 2017)
	Confirmatory		GC-MS	
	Radiovalidation		--	
	ILV			Szuter, 1998 Report no. 34-98-160 DPDB 81853 EU agreed method (RAR, 2017)
Soil	Primary Confirmatory	0.05 mg/kg	LC-MS/MS	Jooß, 2013 Report no. P3051G EU agreed method (RAR, 2017) Applied in report no. 17 48 TEC 0009 18 48 FEW 0001 GOW-17-13 GOW-17-14 18 35 CRX 0033 17 48 TEC 0008 19 48 FEW 0003 GOW-17-3 GOW-17-4
Component(s) analysed: Zoxamide (R, S, sum)				
Soil	Primary Confirmatory	0.002 mg/kg	LC-MS/MS	Kercher, 2017 Report no. AS520

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component(s) analysed: RH-163353 (R, S, sum)				
Soil	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS	Gray, 2020 Report no. 3202389 Report no. 3202389 Report no. 3202391
Component(s) analysed: RH-127450				
Soil	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS	Gray, 2020 Report no. 3202376
Component(s) analysed: RH-24549				
Soil	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS LC-TQ/MS	Gray, 2020 Report no. 3202395
Component(s) analysed: RH-141455				
Soil	Primary Confirmatory	0.2 mg/kg	LC-TOF/MS	Gray, 2020 Report no. 3202382 Report no. 3202383
Component(s) analysed: Zoxamide				
Body fluids and tissues	Primary Confirmatory	0.05 mg/L (body fluids; urine) 0.1 mg/kg (tissues; bovine liver)	LC-MS/MS	Coleman, 2017 Report no. FF/17/002
Drinking water	Primary Confirmatory	0.05 µg/L	GC-ECD GC-MS	Völkl, 1998 Report no. 34-98-52 DPDB 81855 EU agreed method
Drinking water Surface water	Primary Confirmatory	0.05 µg/L	GC-ECD GC-MS	Wais, 2000 Report no. 34-99-201 DPDB 97884 EU agreed method
Salt water	Primary	0.016 mg/L	HPLC-UV	Nixon & Sulaiman (1997) Report no. 129A-136 EU agreed method (RAR, 2017)
Salt water	Primary	20 µg/L	HPLC-UV	Kendall (1998) Report no. 129A-142 EU agreed method (RAR, 2017)
Surface water (*Daphnia*, fish medium)	Primary	0.12 mg/L	HPLC-UV	xxx (2010) Report no. BT102/10 EU agreed method (RAR, 2017)
Salt water (fish)	Primary	0.01 mg/L	HPLC-UV	xxx., 1998 Report no. 97RC-0078
Aquatic media (fish, *Daphnia*, alga)	Primary Confirmatory Method	0.02 µg/L	GC-MS/MS	xxx, 2007 Report no. CH-156/2006 Applied in

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	34-98-5			Report no. CH-E-023/2006 Report no. CH-E-001/2007 Report no. CH-E-002/2007
Aquatic media (*Lemna*)	Primary Confirmatory	0.168 µg/L	HPLC-MS/MS	Juckeland (2019) Report no. 18 48 ALE 0005
Component(s) analysed: RH-141455, RH-141452				
Aqueous buffer solution	Primary	0.05 µg/L	HPLC-DAD	Longhi (2019) Report no. BPL-STUDY-18-000092 Report no. BPL-STUDY-19-00009
	Confirmatory		LC-MS/MS	
Component(s) analysed: RH-163353 (R, S, sum)				
Aquatic media	Primary	0.001 mg/L (Fish) 0.1 mg/L (*Daphnia*, alga, mysid)	LC-TOF/MS	xxx (2020) Report no. 3202385 Jarrome (2020) Report no. 3202386 3202387 3202388
Component(s) analysed: RH-141455				
Aquatic media	Primary Confirmatory	0.25 mg/L (mysid) 0.1 mg/L (Daphnia, fish)	LC-TOF/MS	xxx (2020) Report no. 3202716 Hugill (2019) Report no. 3202380 Hugill (2020) Report no. 3202381
Component(s) analysed: RH-24549				
Aquatic media	Primary Confirmatory	0.1 mg/L (mysid)	LC-TOF/MS LC-TQMS	Hugill (2019) Report no. 3202394
Component(s) analysed: RH-127450				
Aquatic media	Primary Confirmatory	0.01 mg/L	LC-TOF/MS LC-TQMS	xxx (2020) Report no. 32023713 Hugill (2019) Report no. 3202374 Hugill (2019) Report no. 3202375
Component(s) analysed: RH-139432				
Aquatic media	Primary Confirmatory	0.1 mg/L (mysid)	LC-TOF/MS LC-TQMS	Hugill (2019) Report no. 3202398
Component(s) analysed: Zoxamide				
Air	Primary	0.003 mg/m^3	GC-ECD	Wais (1999) Report no. 34-98-51

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Confirmatory		GC-MS	DPDB 81858 EU agreed method

Cymoxanil

For cymoxanil, an overview on the acceptable methods for analysis of residues of cymoxanil for the generation of pre-authorisation data already evaluated at EU level is given in the following table.

Table 5.2-3: Validated methods for the generation of pre-authorisation data cymoxanil

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component analysed: Cymoxanil				
Potato	Primary	0.05 mg/kg	GC-NPD	Freschi, G. 2001 Report no. SIP1277 EU agreed method (DAR 2007)[1]
	Confirmatory	0.05 mg/kg	HPLC-UV	Wasser, C. 2002 Report no.- EU agreed method (DAR 2007)[1]
	ILV	0.05 mg/kg	GC-NPD	Wasser, C. 2002 Report no. A0087 EU agreed method (DAR (2007)[1]
Lettuce	Primary	0.05 mg/kg	GC-NPD	Freschi, G. 2001 Report no. SIP1279 EU agreed method (DAR 2007)[1]
	Confirmatory	0.05 mg/kg	HPLC-UV	Wasser, C. 2002 Report no.- EU agreed method (DAR 2007)[1]
	ILV	0.05 mg/kg	GC-NPD	Wasser, C. 2002 Report no. A0087 EU agreed method DAR (2007)[1]
Animal products, food of animal origin	Not required (DAR 2007)			
Soil	Primary	0.01 mg/kg	HPLC-UV	Melkebeke, T. 2000 Report no. 281802 EU agreed method DAR (2007)[1]
	Confirmatory	0.01 mg/kg	HPLC-DAD	
Drinking water/surface water	Primary Confirmatory	0.10 µg/L	HPLC-UV	Cabusas, M.E.Y. 1999 Report no. DuPont-2126 EU agreed method (DAR 2007)[1]
Air	Primary	0.46 µg/m^3	HPLC-UV	Melkebeke, T. 2000 Report no. 257805 EU agreed method (DAR 2007)[1]
	Confirmatory	0.46 µg/m^3	HPLC-DAD	

[1] Draft Assessment Report – Cymoxanil. Volume 3, Annex B, Part 5. October 2007.

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Body fluids and tissues (Toxicology)	Not required (DAR 2007)			

An overview on methods for analysis of residues of cymoxanil for the generation of pre-authorisation data not yet evaluated at EU level is given in the following table.

Table 5.2-4: Validated methods for the generation of pre-authorisation data cymoxanil

Component analysed: Cymoxanil				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Tomato Canned tomato Puree Juice (high acidic matrix)	Primary B15G-P2-BCZ-01	LOQ 0.01 mg/kg	HPLC-MS/MS	Tetuan, B (2016), report no. 15 F CL GW P/A
Potato	Primary	LOQ 0.01 mg/kg	HPLC-MS/MS	Tetuan, B (2011), report no. 10 F PT GW P/A
Tomato (whole fruit)	Primary	LOQ 0.05 mg/kg	GC/NPD	Freschi, G. (2001) Report n. SIP1278
Grapes (bunches)	Primary	LOQ 0.05 mg/kg	GC/NPD	Freschi, G. (2001) Report n. SIP1276

Cymoxanil

For cymoxanil the following EU concluded methods are available on EU level :

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	GC-NDP: LOQ = 0.04 mg/kg in potato, grape and tomato 0.1 mg/kg hop GC-NPD: LOQ = 0.05 mg/kg in commodities with high water content (potato, lettuce)
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	No residue definition is proposed, therefore no analytical method is required
Soil (analytical technique and LOQ)	HPLC-UV: LOQ = 0.01 mg/kg
Water (analytical technique and LOQ)	HPLC-UV: LOQ = 0.1 µg/L (drinking and surface water) An analytical method for IN-KQ960 in surface with an LOQ lower than 0.3 mg/L and in ground water with an LOQ of 0.1 µg/L is required
Air (analytical technique and LOQ)	HPLC-UV: LOQ = 0.46 µg/m^3
Body fluids and tissues (analytical technique and LOQ)	Cymoxanil is not classified as toxic or highly toxic, therefore no analytical method is required

Zoxamide

For zoxamide, the following EU concluded methods are available :

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	Zoxamide in potato (tuber, chips and flakes), grapes (berries, juice, wine and raisins), lettuce, dry bean and oilseed rape seed: QuEChERS multi-residue method, LC-MS/MS. LOQ: 0.01 mg/kg ILV: Potato tuber, grape vine and lettuce – LOQ 0.01 mg/kg RH-141455 and RH-141452 in potato: LC-MS/MS LOQ: 0.01 mg/kg in potato tubers and 0.05 mg/kg in potato chips and flakes for both metabolites ILV: data gap
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	Pending, data gaps identified
Soil (analytical technique and LOQ)	LC-MS/MS, LOQ: 0.05 mg/kg determining zoxamide

Water (analytical technique and LOQ)	Drinking and surface water: LC-MS/MS, LOQ: 0.1 µg/L determining zoxamide ILV: Drinking water, LOQ: 0.1 µg/L for determining zoxamide
Air (analytical technique and LOQ)	LC-MS/MS, LOQ: 90 µg/m^3
Body fluids and tissues (analytical technique and LOQ)	Data gap

In addition, a new method for the determination of residues of zoxamide in body fluids (urine) and tissues (bovine liver), with QuEChERS extraction procedure and LC-MS/MS detection has been validated according to SANCO 825/00 rev. 8.1 (2010) in Report No. FF/17/002. The method allows the determination of zoxamide in (human) urine and (bovine liver) tissue with an LOQ of 0.05 mg/L and 0.1 mg/kg, respectively.

A new method for the determination of zoxamide and its metabolites via HPLC-MS/MS and HPLC-HRMS/MS in grape fruit, potato tuber, tomato fruit, cucumber fruit, and onion bulb raw agricultural and relevant processed commodities has been developed and provided (Report No. BPL-STUDY-18-000085). It has been fully validated in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The extraction efficiency of the analytical method has been verified according to SANTE 2017/10632 rev. 3.
With this method, it is possible to determine the analytes zoxamide (sum, R/S), RH-150721 (sum, R/S), RH-129151 (sum, R/S), RH-141288 (sum, R/S), RH-127450 (sum, R/S), RH-149736, RH-149737, RH-24549, RH-139432, RH-141452 and RH-141455. All 5 pairs of chiral analytes were separated to receive their single enantiomers by chiral chromatography. For metabolite RH-129151 - due to the lack of single isomer analytical standards - it was impossible to attribute the absolute configuration (R) and (S) to the measured peaks. Therefore, the 2 enantiomers were assigned in the following as "RH-129151 A" (the first enantiomer eluted in the chromatographic runs) and "RH-129151 B" (the second enantiomer eluted in the chromatographic runs). The metabolites RH-141452, RH-141255 and RH-149737, which are known to form sugar conjugates, can be determined after an additional alkaline hydrolysis step according to the QuEChERS method for the simultaneous determination of acidic pesticides, their esters and conjugates following alkaline hydrolysis. It allows to quantitatively hydrolyse of potential conjugates of zoxamide metabolites.
This highly specific HPLC-MS/MS method for the determination of zoxamide and its metabolites in grape, potato, tomato, cucumber, and onion has been validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) at an LOQ of 0.01 mg/kg (for analytes with a chiral centre this limit is referred to the sum of the 2 enantiomers; i.e. the LOQ for each enantiomer is 0.005 mg/kg).

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

For cymoxanil it is referred to the methods available in the RAR (2007)

For zoxamide it is referred to the methods available in the RAR (2017). In addition, it is referred to the data under evaluation in an interzonal procedure with Latvia as RMS and zRMS in this process.

Please note that data requested by EFSA (2017) and in the EC Renewal Report (2017) to conclude on the residue definitions of zoxamide in plant and animal commodities as well as environmental compartments are currently under evaluation in an ongoing interzonal procedure with Latvia as the RMS for zoxamide and inter-zonal RMS for this procedure, together with the relevant countries as cMSs. For final residue definitions together with the underlying data and methods, it is therefore referred to this procedure.

It will be followed by the outstanding review of the maximum residue levels (MRLs) under Article 12 of Regulation (EC) No 396/2005.

5.3.1 Analysis of the plant protection product (KCP 5.2)

Please refer to the analytical methods for the determination of the active substance(s) in the plant protection product as provided in chapter 5.2.1. The method(s) can be applied for post-authorisation and monitoring purposes.

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

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

Soil, sediment, surface water, drinking water and air, body fluids and tissues

Compared to the residue definition proposed by EFSA (2017), the current legal residue definition is not identical.

EFSA (2017) has requested additional toxicological and/or ecotoxicological studies on several zoxamide metabolites and did not conclude on the residue definition for several environmental compartments.

Provisional / proposed residue definitions of EFSA (2017):

Soil	At least zoxamide but open regarding metabolites RH-163353 and RH-141455
Sediment	At least zoxamide but open regarding metabolites RH-127450 and RH-163353
Water surface	At least zoxamide but open regarding RH-127450, RH-24549, RH-163353 & RH-141455
drinking/ground	At least zoxamide but open regarding RH -141455
Air	Zoxamide
Body fluids and tissues	Zoxamide

Soil:

EFSA (2017) proposed as residue definition for soil: "at least zoxamide, but open regarding metabolites RH-163353 and RH-141455".

RH-141455 has been demonstrated to be of lower chronic toxicity to earthworms (NOEC = 5 mg/kg soil d.w.) compared to zoxamide (NOEC = 2.45 mg/kg) (see Table 9.8-1 in the dRR Part B.9). The *Folsomia* and *Hypoaspis* endpoints of zoxamide (each 217 mg a.s./kg soil d.w.) and RH-141455 (each 50 mg a.s./kg soil d.w.) are not comparable since RH-141455 has only been tested up to 50 mg soil d.w. as highest soil concentration; this concentration was sufficient to conclude a safe use for worst-case gap uses of zoxamide. As an additional point, the expected $PEC_{s,accu}$ of RH-141455 in soil are < 10% of the zoxamide PECs. Therefore, it seems not applicable to include RH-141455 in the residue definition for soil, but to keep zoxamide only as relevant (marker) soil residue for monitoring.

RH-163353 has been demonstrated to be of lower chronic toxicity to earthworms (NOEC = 10 mg/kg soil d.w.) compared to zoxamide (NOEC = 2.45 mg/kg) (see Table 9.8-1 in the dRR Part B.9). The *Folsomia* and *Hypoaspis* endpoints of zoxamide are each 17 mg a.s./kg soil d.w., the NOECs for RH-163353 are 4.76 and 27.78 mg a.s./kg soil d.w., respectively. These endpoints are considerably lower than for the parent compound zoxamide. Nevertheless, the $PEC_{s,accu}$ for RH-163353 are again much lower than for zoxamide (1/8 compared to zoxamide). Therefore, it seems not applicable to include RH-163353 in the residue definition for soil, but to keep zoxamide only as relevant (marker) soil residue for monitoring.

Sediment and surface water:

EFSA (2017) proposed the residue definition for surface water as "at least zoxamide, but open regarding RH-127450, RH-24549, RH-163353 and RH-141455". However, as could be demonstrated in the dRR Part B.9 (see Table 9.5-1), all these metabolites show a lower toxicity to aquatic organisms than zoxamide. Therefore, it is proposed to keep zoxamide only as molecule for monitoring of surface water and sediment.

Drinking-/groundwater:

EFSA (2017) proposed the residue definition for drinking water as "at least zoxamide, but open regarding RH-141455". However, as could be demonstrated in the RAR (2017) and in the dRR Part B.6 and B.10, RH-141455 is neither biologically relevant (in terms of efficacy compared to the parent compound) nor toxicologically relevant and does not bear a risk for consumers. Therefore, it is proposed to keep the residue definition for drinking water as zoxamide only.

Air:

EFSA (2017) set the residue definition for air as zoxamide.

Body fluids and tissues:

EFSA (2017) set the residue definition for body fluids and tissues as zoxamide.

<u>Crop and animal matrices for consumer risk assessment</u>

Compared to the residue definition proposed by EFSA (2017), the current legal residue definition is not identical.

According to Reg. (EU) No. 2017/171 the residue definition for monitoring/enforcement in plants is zoxamide only. A no residue situation for zoxamide in commodities of animal origin was concluded in the previous Reg. (EU) No. 520/2011. This changed in Reg. (EU) No. 2017/171, where the MRLs for residues in commodities of animal origin were set to 0.01 mg/kg, defined as zoxamide.

Because of outstanding residue data matching the intended GAP and outstanding questions on the toxicological profile of the zoxamide metabolites RH-141452 and RH-141455, an overall residue definition for

plants was not set during AIR (see EFSA Conclusion, 2017).

For risk assessment, provisional residue definitions were proposed by EFSA (2017): For fruit crops the sum of zoxamide and RH-141452, for pulses and oilseeds zoxamide only, and for root crops the sum of metabolites RH-141455 and RH-141452. For monitoring, the residue definitions were proposed as zoxamide for fruit crops, pulses and oilseed (as marker), and the metabolites RH-141455 and RH-141452 for root crops.

For commodities of animal origin, the residue definition mentioned by EFSA (2017) is "open".
For GWN-10392 the intended cops are grape and tomato. Grape and tomato pomace are not fed to livestock. New trials are available to support the potato uses on EU level. These trials demonstrate no residues > LOQ (0.01 mg/kg) of zoxamide and its potentially relevant metabolites (RH-141455 and RH-141452) in potato tubers. Besides, additional data are available to support the toxicologically non relevance of the structurally related metabolites RH-141452 and RH-141455. As a result, livestock is not deemed to be exposed to residues of zoxamide via feed above the trigger value established in Reg. (EC) No 1107/2009. The MRLs for zoxamide in commodities of animal origin – as laid down in Reg. (EU) No. 2017/171 (i.e. 0.01 mg/kg) - do not need to be changed within the course of this application.

Provisional / proposed residue definitions of EFSA (2017) – raw agricultural crop commodities:

Plant residue definition for monitoring (RD-Mo) OECD Guidance, series on pesticides No 31	Zoxamide (fruit, pulses and oilseeds) Metabolites RH-141455 and RH-141452 (root crops) pending data gap for RH-141455 and RH-141452
Plant residue definition for risk assessment (RD-RA)	Zoxamide and RH 141452 (fruit) pending data gap on RH-141452 Zoxamide (Pulses and oilseeds) Metabolites RH-141455 and RH-141452 (root crops) pending data gap for RH-141455 and RH-141452
Conversion factor (monitoring to risk assessment)	1

Provisional / proposed residue definition of EFSA (2017) – commodities of animal origin:

Food of animal origin	Open

The zoxamide metabolites RH-141452 and RH-141455 were identified as major metabolites (>10% TRR) in the potato metabolism study (Reibach, PH and Spencer, WO, 1998) but are not found in actual supervised field residue trials at levels ≥ 0.01 mg/kg.

In the metabolism studies on fruit crops, zoxamide was the main component of the total radioactive residue (TRR) with 48% and 44% in green and red tomatoes and 92% in both cucumber and grape. The remaining TRR was extensively metabolised to a range of degradation products, representing each less than 10% TRR. Also, RH-141452 in green and red tomato fruits was shown to occur << 10% TRR in the RAR (2017) – even if its conjugated forms are taken into account (RH-141452 occurred both free and as (mainly glucose) conjugate in tomato fruits). New supervised residue trials confirmed that RH-141452 does not occur at levels above 0.01 mg/kg in tomato fruits when applied at the intended application pattern. However, it occurs at or slightly above the LOQ (max. of 0.0188 mg/kg) in grape fruits. But the new toxicological studies requested by EFSA (2017) confirmed that the structurally related RH-141455 and RH-145452 are not of toxicological relevance. As a result, this metabolite should not be included in the residue definition for enforcement and risk assessment of fruit crops. Instead, it is proposed to keep zoxamide in the residue definition for enforcement.

Following foliar application of ^{14}C-labelled zoxamide to primary crops, most of the applied material generally remains on the plant surface. In the metabolism studies conducted on grapes, tomatoes, cucumbers and peas, the major component of the residue was unchanged zoxamide (RH-7281).

Moreover, a similar degradation path of zoxamide in crops can be concluded, with zoxamide as main / marker residue. However, in contrast to e.g. pulses, vines or tomatoes, different uptake paths via potato tubers and/or their roots of probably the soil metabolites of zoxamide seem to play a role when defining zoxamide residues after spray application to bulb and tuber crops (e.g. onions, potatoes).

As zoxamide is a racemate, its metabolites containing the chiral carbon atom may feature two enantiomers. Chiral analysis of residues confirmed the stability of the chiral centre of zoxamide and its metabolites. As a result, it is applicable to consider the residues of zoxamide and its metabolites as sum of enantiomers.

Crop processed commodities

Based on the currently available data, the residue definition for risk assessment of plant processed commodities is currently open / tentatively with regard to

- RH-150721 in grape and tomato processed fractions; separate ADI of 0.56 mg/kg bw/d (see dRR Part B.7 and B.6)
- Zoxamide +…

  RH-129151 in tomato processed fractions

  RH-24549 in grape and tomato processed fractions

  RH-141288 in raisins

Please note that data requested by EFSA (2017) and in the EC Renewal Report (2017) to conclude on the residue definitions of zoxamide in plant and animal commodities as well as environmental compartments are currently under evaluation in an ongoing interzonal procedure with Latvia as the RMS for zoxamide and inter-zonal RMS for this procedure, together with the relevant countries as cMSs. For final residue definitions together with the underlying data and methods, it is therefore referred to this procedure.

It will be followed by the outstanding review of the maximum residue levels (MRLs) under Article 12 of Regulation (EC) No 396/2005.

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Potato tuber, potato chips, potato flakes, raisins, grape berries, juice and wine, lettuce, dry bean, oilseed rape seed Representing commodities of high-water content, acidic, high oil content and dry	Zoxamide	0.01 mg/kg	Weber, 2012, report no. S12-03949 Richter, 2014, report no. P3114G Schlewitz, 2014, report no. R B4023 EU agreed method (QuEChERS) (RAR 2017)
Plant, difficult matrices (hops, spices, tea)		--	--
Muscle	Not relevant (tentative)	0.01 mg/kg	Reg. (EU) 2017/171
Milk		0.01 mg/kg	Reg. (EU) 2017/171

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Eggs		0.01 mg/kg	Reg. (EU) 2017/171
Fat		0.01 mg/kg	Reg. (EU) 2017/171
Liver, kidney		0.01 mg/kg	Reg. (EU) 2017/171
Soil (Ecotoxicology)	Zoxamide	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Zoxamide	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Zoxamide	LOQ = 0.1 µg/L	Jooß, 2013 Report no. P3050G EU agreed method (RAR, 2017) This covers the lowest aquatox endpoint of 3.48 µg/L (fish, chronic).
Air	Zoxamide	90 µg/m^3	Miller, 2013 Report no. FRK0048 EU agreed method (RAR, 2017) $AOEL_{syst.}$ = 0.3 mg/kg bw/d
Tissue (bovine liver)	Zoxamide	0.1 mg/kg	Coleman, 2017 Report no. FF/17/002
Body fluids		0.05 mg/L	

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 zoxamide residues in plant matrices is given in the following tables. For the detailed evaluation of new/additional studies it is referred to Appendix 2.

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: zoxamide				
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 Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory (also for R/S ratio)	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G 10186-01-VMPL
	ILV	0.01 mg/kg	LC-MS/MS	Schlewitz, 2014

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
			(QuEChERS)	report no. R B4023 EU agreed method (RAR 2017)
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 report no. BPL-STUDY-18-000085
High acidic content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory (also for R/S ratio)	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G 10186-01-VMPL
	ILV	0.01 mg/kg	LC-MS/MS (QuEChERS)	Schlewitz, 2014 report no. R B4023 EU agreed method (RAR 2017)
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 report no. BPL-STUDY-18-000085
High oil content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory (also for R/S ratio)	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G 10186-01-VMPL
	ILV	0.01 mg/kg	LC-MS/MS (QuEChERS)	Schlewitz, 2014 report no. R B4023 EU agreed method (RAR 2017)
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 report no. BPL-STUDY-18-000085
High protein/high starch content (dry)	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter , 2014 report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory (also for R/S	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G 10186-01-VMPL

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
	ratio)			
	ILV	0.01 mg/kg	LC-MS/MS (QuEChERS)	Schlewitz, 2014 report no. R B4023 EU agreed method (RAR 2017)
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala , 2020 report no. BPL-STUDY-18-000085

The extraction efficiency

The efficiency of the extraction procedure (QuEChERS) used in the monitoring methods detailed above was demonstrated using radio-labelled samples from the pea metabolism study (see RAR 2017, CA 6.2.1/08). Samples of immature whole plant and dry peas containing incurred residues of zoxamide were extracted with acetonitrile and a salt solution in accordance with the QuEChERS method. The organic extracts were profiled by radio-TLC and HPLC, and the profiles compared with those obtained in the metabolism study. The amount of available zoxamide in the samples, determined in the pea metabolism study, was 0.449 mg/kg for immature whole plant and 0.019 mg/kg for dry peas. The amount of zoxamide extracted using the QuEChERS extraction method was 0.0442 mg/kg for immature whole plant and 0.019 mg/kg for dry peas, giving recoveries of 98.4% and 68.4% for immature whole plant and dry peas respectively.

EFSA (2017) has requested *"A fully validated monitoring method for RH-141455 and RH-141452 in potatoes (relevant for the representative uses in potato; submission date proposed by the applicant: unknown; see Section 1)."* This method is provided (Witte, 2020; report no. 18G10186-01-VMPL).

With regard to the provisional residue definition of EFSA (2017) on root and tuber crops, the following methods are available.

Table 5.3-3: 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: RH-141455, RH-141452				
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 Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G10186-01-VMPL
High acid content	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G10186-01-VMPL
High oil content	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 Report no. 18G10186-01-VMPL
High protein/high starch content (dry)	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 report no. 18G10186-01-VMPL
High water content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS	Sala, 2020 report no. BPL-STUDY-18-000085
High acid content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS	Sala, 2020 report no. BPL-STUDY-18-000085
High protein/high starch content (dry)	Primary Confirmatory	0.01 mg/kg	LC-MS/MS	Sala, 2020 report no. BPL-STUDY-18-000085

5.3.2.3 Description of analytical methods for the determination of residues in animal

matrices (KCP 5.2)

No new data. / No relevant for this submission.

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

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

Table 5.3-4: Validated methods for soil

Component of residue definition: zoxamide			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary Confirmatory	0.05 mg/kg	LC-MS/MS	Jooß, 2013 report no. P3051G EU agreed method (RAR, 2017)

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 zoxamide in surface and drinking water is given in the following tables.

Table 5.3-5: Validated methods for water (if appropriate)

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Surface and drinking water	Primary Confirmatory	0.1 µg/L	LC-MS/MS	Jooß, 2013 report no. P3050G EU agreed method (RAR, 2017)
	ILV			Schlewitz, 2014 report no. RB4049 EU agreed method (RAR, 2017)

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

No new data. / No relevant for this submission.

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

EFSA (2017) has requested *"An analytical method for monitoring zoxamide in body fluids and tissues (relevant for all representative uses evaluated; submission date proposed by the applicant: unknown; see Section 1)."* This method is provided.

An overview on the acceptable methods for analysis of zoxamide in body fluids and tissues is given in the following table. For the detailed evaluation of new / additional studies it is referred to Appendix 2.

Table 5.3-6: Methods for body fluids and tissues (if appropriate)

Component of residue definition: zoxamide			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary Confirmatory	0.05 mg/L (body fluids; urine) 0.1 mg/kg (tissues; bovine liver)	LC-MS/MS	Coleman, 2017 Report no. FF/17/002

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

5.3.2.8 Other studies/ information

No new data. / No relevant for this submission.

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

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

Compared to the residue definition proposed in the Draft Assessment Report (incl. its addenda) the current legal residue definition is identical.

Table 5.3-7: 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 water content	Cymoxanil	LOQ: 0.05 mg/kg	EFSA Scientific Report (2008) 167, 1-116
Plant, high acid content		LOQ: 0.04 mg/kg	EFSA Scientific Report (2008) 167, 1-116
Plant, high protein/high starch content (dry commodities)		--	
Plant, high oil content		--	--
Plant, difficult matrices (hops, spices, tea)		LOQ: 0.1 mg/kg	EFSA Scientific Report (2008) 167, 1-116
Muscle	Not applicable	--	--
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	Cymoxanil	0.01 mg/kg	EFSA Scientific Report (2008) 167, 1-116
Drinking water (Human toxicology)	Cymoxanil IN-KQ960	0.1 µg/L	EFSA Scientific Report (2008) 167, 1-116
Surface water (Ecotoxicology)			
Air	Cymoxanil	0.46 µg/m^3	EFSA Scientific Report (2008) 167, 1-116
Tissue (meat or liver)	Not applicable	--	Not classified as T / T+
Body fluids			

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 for analysis of cymoxanil in plant matrices is given in the following tables.

Table 5.3-8: 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: Cymoxanil				
Matrix type	**Method type**	**Method LOQ**	**Principle of method**	**Author(s), year / missing**
High water content (potato and tomato)	Primary	0.05 mg/kg	GC-NPD	EFSA Scientific Report (2008) 167, 1-116
High acid content (grape)				
Difficult (hop)	Primary	0.01 mg/kg		
High water content (potato and lettuce)	Primary	0.05 mg/kg		

Table 5.3-9: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	-

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

Not applicable.

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

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

Table 5.3-10: Validated methods for soil

Component of residue definition: cymoxanil			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.01 mg/kg	HPLC-UV	EFSA Scientific Report (2008) 167, 1-116

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 cymoxanil in surface and drinking water is given in the following tables.

Table 5.3-11: Validated methods for water

Component of residue definition: Cymoxanil				
Matrix type	**Method type**	**Method LOQ**	**Principle of method**	**Author(s), year / missing**
Drinking and surface water	Primary	0.1 µg/L	HPLC-UV	EFSA Scientific Report (2008) 167, 1-116
Water (pond, stream, well and tap)	Primary	0.100 ppb	HPLC/MS-MS	Leak T., 2010
Water (drinking and steam)	ILV	0.100 ppb	HPLC/MS-MS	Cermak J., 2013
Component of residue definition: IN-KQ960				
Water (pond, stream, well and tap)	Primary	0.100 ppb	HPLC/MS-MS	Leak T., 2010
Water (drinking and steam)	ILV	0.100 ppb	HPLC/MS-MS	Cermak J., 2013

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 cymoxanil in air is given in the following tables.

Table 5.3-12: Validated methods for air

Component of residue definition: Cymoxanil			
Method type	**Method LOQ**	**Principle of method**	**Author(s), year / missing**
Primary	0.46 µg/m^3	HPLC-UV	EFSA Scientific Report (2008) 167, 1-116

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

No analytical methods are required as Cymoxanil is not classified as toxic or highly toxic.

5.3.3.8 Other studies/ information

None

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and 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
KCP 5.1.1	Diogo, A.	2003	Analytical method and validation for the determination of cymoxanil and zoxamide in Harpon XF – 98083 formulation Gowan Crop Protection Ltd., UK Dow AgroSciences Industrial Ltda., Brazil, Report no. DAS–AM–02–051 GLP Not published	N	GWI Sipcam Oxon S.p.A.
KCP 5.1.1/02	De Ryckel, B.	2019	Validation of analytical method of cymoxanil and zoxamide content and Physico-chemical properties and storage stability of Cymoxanil 33% + zoxamide 33% WG Gowan Crop Protection Ltd., UK Walloon Agricultural Research Centre (CRA-W), Belgium, Report No. 24718 GLP No Published	N	GWI
KCP 5.1 (KCA 4.1)	Peterek, S.	2020	Magnitude of the residues of zoxamide and its metabolites in grapevine (RAC bunches) and processed fractions, following applications of Zoxium 240 SC, Northern Europe – 2018 Gowan Crop Protection Ltd., UK Staphyt GmbH, Germany, Report No. AB2-18-35355 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Sala, A.	2020	Determination of zoxamide and its metabolites in raw agricultural commodity wine grape (berries) and processed fractions (juice, wine) following five applications of Zoxium 240 SC (GWN-9790 EU) in open field condition, 2 harvest trials, Northern Europe, year 2017 – final report amendment no. 1 Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000041 GLP	N	GWI

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
			Not published ⇨ Filed under KCA 6.3		
KCP 5.1 (KCA 4.1)	Thomas-Delille, E.	2020	Determination of zoxamide and its metabolite RH-150721 residues in wine grape and processed fractions following five foliar applications with Zoxium 240 SC under field conditions in Northern Europe in 2017 – amended final report Gowan Crop Protection Ltd., UK Anadiag, France, Report No. B7284 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Sala, A.	2020	Determination of zoxamide and its metabolites in raw agricultural commodity wine grape (berries) and processed fractions (juice, wine) following five applications of Zoxium 240 SC (GWN-9790 EU) in open field condition 2 harvest trials, Southern Europe, year 2017 - final report amendment no. 1 Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000051 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Casalinuovo, L.	2020	Determination of zoxamide and his metabolite RH-150721 residues in raw agricultural commodity red grapes and processed fraction following five applications of Zoxium 240 SC (zoxamide 240 g/L) (South Europe - 2 trials year 2017) plus amendment no. 1 to final report Gowan Crop Protection Ltd., UK Biotecnologie B.T., Italy, Report No. BIU-005-17 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Longhi, D.	2020	Determination of zoxamide and its metabolites in raw agricultural commodity of grape wine in open field following five and three applications of the formulated product GWN 9790 EU (North Europe - 4 trials year 2019) Gowan Crop Protection Ltd., UK	N	GWI

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
			LabAnalysis, Italy, Report No. BPL-STUDY-19-000057 GLP Not published ⇨ Filed under KCA 6.3		
KCP 5.1 (KCA 4.1)	Longhi, D.	2020	Determination of zoxamide and its metabolites in raw agricultural commodity of table grape and processed (raisin) in open field following five and three applications of the formulated product GWN 9790 EU (South Europe – 1 trial year 2019) Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000058 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Maccaferri, L.	2020	Magnitude of the residues of zoxamide in table grape bunches and in raisins processed fraction, following applications of Zoxium 240 SC. One harvest trial, Southern Europe – 2018 Gowan Crop Protection Ltd., UK Renolab S.r.l., Italy, Report No. 18097-03R GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Maccaferri, L.	2019	Determination of the residues of zoxamide and/or phosphorous acid in table grape raw agricultural commodity following five applications of GOW F 716, Zoxium 240 SC, GOW F 316 in open field conditions (one harvest trial, Italy 2017) Gowan Crop Protection Ltd., UK Renolab S.r.l., Italy, Report No. 17120-01R, GLP, Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Maccaferri, L.	2019	Determination of the residues of zoxamide and/or phosphorous acid in raw agricultural commodity of grape-vine and processed commodities (juice, must, young wine and bottled wine) following five applications of GOW F 716, Zoxium 240 SC, GOW F 316 in open field conditions (one harvest trial, Italy 2017) Gowan Crop Protection Ltd. UK Renolab S.r.l., Italy, Report No. 17120-02R, GLP, Not published	N	GWI

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
			⇨ Filed under KCA 6.3		
KCP 5.1 (KCA 4.1)	Maccaferri, L.	2020	Magnitude of residues of zoxamide enantiomers and metabolites in grapes and processed commodities (juice, must, young wine and bottled wine) following five applications of GOW F 716 and Zoxium 240 SC in open field condition (Italy 2017) Gowan Crop Protection Ltd., UK Renolab S.r.l, Italy, Report No. 19200-01R GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Luciani, G.P.	2016	Determination of zoxamide and benalaxyl-m residues after three applications of GWN-10392 on wine grapes under field conditions – Italian trial, year 2015 Gowan Comercio Internacional et Servicos Lda., Portugal Tentamus AgriParadigma S.r.l., Italy, Report No. AGRI 009/15 GLP HAR GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Perboni, A.	2017	Determination of benalaxyl-m and zoxamide residues in raw agricultural commodity grapes (wine and table) and processed commodity (must, fermenting must, wine and aged wine) following three applications of GWN-10392 (benalaxyl-m 150 g/L + zoxamide 225 g/L) in open field condition (3 harvest trials, Northern and Southern Europe, year 2015) Gowan Crop Protection Ltd., UK Biotecnologie BT, Italy, Report No. RAU-049-15 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Romanini, M	2011	Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) Following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Italy 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal and Oxon Italia S.p.A., Italy Research Centre "E. Gagliardini", Italy, Report No. CREG2117	N	GWI Sipcam Oxon S.p.A.

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
			GLP Not published ⇨ Filed under KCA 6.3		
KCP 5.1	Romanini, M.	2011	Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Northern Europe 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal and Oxon Italia S.p.A., Italy Research Centre "E. Gagliardini", Italy, Report No. CREG2120 GLP Not published ⇨ Filed under KCA 6.3	N	GWI Sipcam Oxon S.p.A.
KCP 5.1 (KCA 4.1)	Longhi, D.	2020	Determination of (R) and (S) zoxamide residues and its metabolites RH-150721, RH-129151, RH-141452, RH-141288, RH-24549 in raw agricultural commodity of industrial tomato and its processed products (juice, puree and peeled tomatoes) following five applications of formulated product Zoxium 240 SC (sponsor code GWN-9790 EU) in open field (South Europe – 4 trials years 2018) Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-18-000014 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Longhi, D.	2020	Determination of zoxamide and its metabolites in raw agricultural commodity of industrial tomato in open field following five applications of the formulated product GWN 9790 EU (South Europe - 4 trials year 2019) Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000059 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Tetuan, B.	2016	Determination of residues at harvest of zoxamide, benalaxyl-m and cymoxanil in tomato, following three broadcast applications of GWN-10392, GWN-9823 and IR6141-copper oxychloride-copper hydroxide 5-15-15 WG under greenhouse conditions and determination of residues at harvest of zoxamide and benalaxyl-m in	N	GWI

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
			industry tomato and its processed products (canned tomatoes, puree and juice), following three broadcast applications of GWN-10392 under open field conditions - South Europe - season 2015 Gowan Comercio Interncional et Servicos Ltd., Portugal Promovert Crop Services SL, Spain, Report No. 15 F CL GW P/A GLP Not published ⇨ Filed under KCA 6.3		
KCP 5.1	Romanini, M.	2011	Determination of cymoxanil and zoxamide residues at harvest in raw and processed commodity tomato (fruit, juice, puree and canned) following five applications of Harpon WG (cymoxanil 33% + zoxamide 33% WG) - four trials, Italy 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal and Oxon Italia S.p.A. CREG, Research Centre "E. Gagliardini", SIPCAM S.p.A., 26857 Salerano sul Lambro (LO), Italy, Report no: CREG2118 GLP Not Published ⇨ Filed under KCA 6.3	N	GWI Sipcam Oxon S.p.A.
KCP 5.1	Devine, H.C.	2006	Residues of Mancozeb and zoxamide in field and protected tomatoes at intervals and at harvest following multiple applications of Electis, Northern France and the United Kingdom – 2006 Dow Agrosciences, UK CEM Analytical Services Ltd (CEMAS), UK, Report No. GHE-P-11604, CEMS-2967 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Tetuan, B.	2011	Determination of residues at harvest in potatoes, following six broadcast applications of Harpon WG, under field conditions - Northern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/A, PROMO/ZOX-CYM/10.01 GLP Not published ⇨ Filed under KCA 6.3	N	GWI

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	Tetuan, B.	2011	Determination of residues at harvest in potatoes, following five broadcast applications of HARPON WG, under field conditions - Southern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/ B, PROMO/ZOX-CYM/10.01 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Terranegra, A.	2020	Magnitude of residue of zoxamide and metabolite RH-1452 and RH-1455 in potatoes (RAC tubers) and processed fractions, following 5 applications of GWN 9790 EU in two trials (2 HS), Northern Europe (France and Poland) – 2017 – amended final report GOWAN Crop Protection Ltd., UK Staphyt Italia S.r.l., Italy, Report No. ATA-18-30694, BPL-STUDY-19-0000065 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Pandolfi, A.	2020	Determination of the residues of zoxamide (R), (S) and sum and its metabolites in raw agricultural commodity potato (tubers) and its processed fractions (chips, baked/cooked, fried and flakes) following five applications of Zoxium 240 SC (sponsor code GWN-9790 EU) in open field condition (Italy - Southern Europe - 2 trials year 2018) Gowan Grop Protection Ltd., UK Res Agraria S.r.l., Italy, Report No. RA 18 051 BPL GW, BPL-STUDY-19-000025 GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Sala A.	2021	Interim Report – Storage stability of Zoxamide residues under frozen conditions (-18°C) in potato tubers, potato flakes and fried potatoes Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-18-000047 GLP Not published ⇨ Filed under KCA 6.1	N	GWI

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 (KCA 4.1)	Luciani, G.P.	2012	Determination of zoxamide and dimethomorph residues after two applications of Zoxium 240 SC and GWN-9963 on lettuce, rocket salad and endive under field conditions - Italian trial year 2012 Gowan Italia Spa, Italy AgriParadigma Srl, Italy, Report No. AGRI 013/12 GLP DEC GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1 (KCA 4.1)	Luciani, G.P.	2012	Determination of zoxamide and dimethomorph residues after two applications of Zoxium 240 SC and GWN-9963 on lettuce and rocket - Italian trial, year 2012 Gowan Italia Spa, Italy AgriParadigma Srl, Italy, Report No. AGRI 014/12 GLP DEC GLP Not published ⇨ Filed under KCA 6.3	N	GWI
KCP 5.1	Longhi D.	2021	Evaluation of the stability of the analyte RH-129151 in the final extract of the following commodities and processed: grape, grape juice, wine, raisin, potato, fried potato, potato fries, tomato, peeled tomato, cucumber Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. GLP-STUDY-20-77 GLP Not published ⇨ Filed under KCA 6.1	N	GWI
KCP 5.1 (KCA 4.1)	Longhi, D.	2019	RH-141452: Hydrolysis under simulated processing conditions Gowan Crop Protection Ltd., UK LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-18-000092 GLP Not published ⇨ Filed under KCA 6.5.1	N	GWI
KCP 5.1 (KCA 4.1)	Longhi, D.	2019	RH-141455: Hydrolysis under simulated processing conditions Gowan Crop Protection Ltd., UK LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-19-000009	N	GWI

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
			GLP Not published ⇨ Filed under KCA.6.5.1		
KCP 5.1 (KCA 4.1)	Cashmore, A.	2019	RH-141288: Partition coefficient (n-octanol/water) Shake flask method Gowan Crop Protection Ltd., UK Smithers ERS Ltd, Uk, Report No. 3202371 GLP Not published ⇨ Filed under KCA.6.7.1	N	GWI
KCP 5.1 (KCA 4.1)	xxx	2020	RH-163353: Fish, acute toxicity test Gowan Crop Protection Ltd., UK xxx, Report No. 3202385 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI
KCP 5.1 (KCA 4.1)	Jarrom, R.	2020	RH-163353**:** Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202386 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Jarrom, R.	2020	RH-163353: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202387 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Jarrom, R.	2020	RH-163353: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ESG Ltd., UK, Report No. 3202388	N	GWI

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
			GLP Not published ⇨ Filed under KCP 10.2.1		
KCP 5.1 (KCA 4.1)	xxx	2020	RH-141455: Fish, acute toxicity test Gowan Crop Protection Ltd., UK xxx, , Report No. 3202716 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI
KCP 5.1 (KCA 4.1)	Hugill, E.	2020	RH-141455: Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202380 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Hugill, E.	2020	RH-141455: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202381 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	xxx	2020	RH-127450: Fish, acute toxicity test Gowan Crop Protection Ltd., UK xxx Report No. 3202373 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI
KCP 5.1 (KCA 4.1)	Hugill, E.	2019	RH-127450: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202374	N	GWI

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
			GLP Not published ⇨ Filed under KCP 10.2.1		
KCP 5.1 (KCA 4.1)	Hugill, E.	2019	RH-127450: Inhibition of growth on the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202375 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Hugill, E.	2019	RH-24549: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202394 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Hugill, E.	2019	RH-139432: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK Report No. 3202398 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1 (KCA 4.1)	Juckeland, D.	2018	Effects of Zoxium 240 SC on *Lemna gibba* in a growth inhibition test under semi-static test conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 18 48 ALE 0005 GLP Not published ⇨ Filed under KCP 10.2.1	N	GWI
KCP 5.1	xxx.	2007	Cymoxanil 33% + Zoxamide 33% WG: validation of the analytical method for the determination of the content of zoxamide in water samples from the aquatic ecotoxicological studies Oxon Italia S.p.A., Italy	N	GWI

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
			xxx, Report No. CH-156/2006 GLP Not published		Sipcam Oxon S.p.A.
KCP 5.1	xxx	2007	Acute toxicity of Cymoxanil 33% + Zoxamide 33% WG to rainbow trout (*Oncorhynchus mykiss*), determined under flow-through conditions Oxon Italia S.p.A., Italy xxx, Report No. CH-E-023/2006 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI Sipcam Oxon S.p.A.
KCP 5.1	xxx	2007	Acute toxicity of Cymoxanil 33% + Zoxamide 33 % WG to *Daphnia magna* in a 48-hour immobilization test under semi-static exposure-limit test Oxon Italia S.p.A., Italy xxx, Report No. CH-001/2007 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI Sipcam Oxon S.p.A.
KCP 5.1	xxx	2007	Toxicity of Cymoxanil 33% + Zoxamide 33% WG to green algae *Pseudokirchneriella subcapitata* determined in a growth inhibition study Oxon Italia S.p.A., Italy xxx, Report No. CH-002/2007 GLP Not published ⇨ Filed under KCP 10.2.1	Y	GWI Sipcam Oxon S.p.A.
KCP 5.1 (KCA 4.1)	xxx	2020	xxx, 2020: Final report addendum for RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) Gowan Crop Protection Ltd., UK xxx, Report No. 129A-143A GLP Not published	N	GWI

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
			⇨ Filed under KCP 10.2.2		
KCP 5.1 (KCA 4.1)	xxx	1998	RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) xxx, Report No. 97RC-0078 Gowan Crop Protection Ltd., UK xxx, Report No. 129A-143A GLP Not published ⇨ Filed under KCP 10.2.2	Y	GWI
KCP 5.1 (KCA 4.1)	Kercher, S.	2017	Enantioselective degradation of (R)-zoxamide and (S)-zoxamide in one soil incubated under aerobic conditions Gowan Crop Protection Ltd., UK RLP AgroScience GmbH, Germany, Report No. AS520 GLP Not published ⇨ Filed under KCP 9.1.1.1	N	GWI
KCP 5.1 (KCA 4.1)	Friedrich, S.	2018	Effects of Zoxium 240 SC on the reproduction of the earthworm *Eisenia andrei* in artificial soil with 5 % peat content Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 17 48 TEC 0009 GLP Not published ⇨ Filed under KCP 10.4.1.1	N	GWI
KCP 5.1 (KCA 4.1)	Schulz, L.	2020	Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK, BioChem agrar, Germany, Report No.18 48 FEW 0001 GLP Not published ⇨ Filed under KCP 10.4.1.2	N	GWI
KCP 5.1	Parsons, Ch	2020	Zoxium 240 SC - A laboratory test to determine the effects of fresh residues on the springtail *Folsomia*	N	GWI

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)			*candida* (Collembola, Isotomidae) in artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, Report No. GOW-17-13 GLP Not published ⇨ Filed under KCP 10.4.2		
KCP 5.1 (KCA 4.1)	Parsons, Ch	2020	Zoxium 240 SC – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, Report No. GOW-17-14 GLP Not published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1	Thomas, H.	2020	Validation of a HPLC-MS-MS method for the determination of cymoxanil and zoxamide in soil Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 18 35 CRX 0033 GLP Not Published	N	GWI
KCP 5.1	Friedrich, S.	2020	Effects of Cymoxanil 33 % + Zoxamide 33 % WG on the reproduction of the earthworm *Eisenia andrei* in artificial soil Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 17 48 TEC 0008, 18 35 CRX 0029 GLP Not Published ⇨ Filed under KCP 10.4.1.2	N	GWI
KCP 5.1	Schulz, L.	2020	Effects of Cymoxanil 33% + Zoxamide 33% WG on earthworms under field conditions Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 19 48 FEW 0003, 19 35 CRX 0030 GLP Not published	N	GWI

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
			⇨ Filed under KCP 10.4.1.2		
KCP 5.1	Parson, Ch.	2020	Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-3, 18 35 CRX 0026 GLP Not Published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1	Parson, Ch.	2020	Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-4; 18 35 CRX 0027 GLP Not published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J.	2020	RH-163353: Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No.3202389 GLP Not published ⇨ Filed under KCP 10.4.1.1	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J.	2020	RH-163353: Collembolan reproduction test in soil Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202390 GLP Not published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J	2020	RH-163353: Effect on reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202391	N	GWI

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
			GLP Not published ⇨ Filed under KCP 10.4.2		
KCP 5.1 (KCA 4.1)	Gray, J.	2020	RH-127450: Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Ltd, UK, Report No.3202376 GLP Not published ⇨ Filed under KCP 10.4.1.1	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J.	2019	RH-24549: Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No.3202395 GLP Not published ⇨ Filed under KCP 10.4.1.1	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J.	2020	RH-141455: Collembolan reproduction study Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202382 GLP Not published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1 (KCA 4.1)	Gray, J.	2020	RH-141455: Effect on reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202383 GLP Not published ⇨ Filed under KCP 10.4.2	N	GWI
KCP 5.1 (KCA 4.1)	xxx	2020	RH-141455: 90-day oral dietary toxicity study with toxicokinetics and 28-day recovery period in Sprague Dawley rats Gowan Crop Protection Ltd., UK	Y	GWI

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
			xxx, Report No. U-19102 GLP Not published ⇨ Filed under KCP 7.4		
KCP 5.1 (KCA 5.8.1)	xxx	2020	RH-141455: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats Gowan Crop Protection Ltd, UK xxx, Report No. U-19044 No GLP Not published ⇨ Filed under KCP 7.4	Y	GWI
KCP 5.1 (KCA 5.8.1)	xxx	2020	RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats Gowan Crop Protection Ltd, UK xxx, Report No. U-19071 No GLP Not published ⇨ Filed under KCP 7.4	Y	GWI
KCP 5.1 (KCA 4.1)	xxx	2020	RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats Gowan Crop Protection Ltd., UK xxx, Report No. U-19134 No GLP Not published ⇨ Filed under KCP 7.4	Y	GWI
KCP 5.1 (KCA 4.1)	xxx	2020	Analytical method validation for the estimation of RH-150721 in rat feed by reverse phase high performance Liquid Chromatography Gowan Crop Protection Ltd., UK xxx, Report No. U-19162 GLP Not published	N	GWI
KCP 5.1	xxx	2019	RH-141455: Analytical method validation for the estimation of RH-141455 in rat feed by reverse phase high performance liquid chromatography was used for the dose formulation analysis	N	GWI

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)			Gowan Crop Protection Ltd., UK xxx, Report No. U-19069 GLP Not published		
KCP 5.1 (KCA 4.1)	Picard, Ch.R.	2018	Zoxamide: Honey bee (*Apis mellifera* L.) larval toxicity, repeated exposure Exigent LLC, A Gowan Group Company, USA Smithers Viscient, USA, Report No. 12791.6307 GLP Not published ⇨ Filed under 10.3.1.3	N	GWI
KCP 5.1	Ruhland, S.	2018	Chronic toxicity of Cymoxanil 33% + Zoxamide 33% WG to the honey bee *Apis mellifera* L. under laboratory conditions Gowan Crop Protection Ltd., UK and Oxon Italia S.p.A, Italy BioChem agrar, Germany, Report No. 17 48 BAC 0005, 17 35 CRB 0009 GLP Not Published ⇨ Filed under KCP 10.3.1.2	N	GWI Sipcam Oxon S.p.A.
KCP 5.1	Scheller, K.	2020	Cymoxanil 33% + Zoxamide 33% WG - Repeated exposure of honeybee (*Apis mellifera* L.) larvae under laboratory conditions (*in vitro*) Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 17 48 BLC 0005, 17 35 CRB 0010 GLP Not published ⇨ Filed under KCP 10.3.1.3	N	GWI Sipcam Oxon S.p.A.
KCP 5.1	Schnurr, A.	2020	Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 18 48 BFB 0001, 18 35 CRB 0040 GLP Not published	N	GWI Sipcam Oxon S.p.A.

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
			⇨ Filed under KCP 10.3.1.6		
KCP 5.1	Schnurr, A.	2020	Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions in Spain (Southern zone) with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK and SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 19 48 BFB 0001, 18 35 CRB 0086 GLP Not published ⇨ Filed under KCP 10.3.1.6	N	GWI Sipcam Oxon S.p.A.
KCP 5.2	Witte, A.	2020	Validation of an analytical method for the determination of residues of R/S-isomers of zoxamide and metabolites RH-141452 and RH-141455 in 4 different matrices: potato tubers (water containing matrix), potato flakes (dry matrix), potato chips (fat containing matrix) and pickled silver skin onions (acidic matrix) Gowan Crop Protection Ltd., UK CIP Pforzheim, Germany., Report No. 18G10186-01-VMPL GLP Not published	N	GWI
KCP 5.2 (KCA 4.2)	Sala, A.	2020	Validation of an analytical method to determine zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-18-000085 GLP Not published	N	GWI
KCP 5.2	Poráczki, K.	2020	Magnitude of residues of zoxamide in *Phacelia* (*Phacelia tanacetifolia* BENTH.) honey after three applications of GWN-9790EU under semi-field conditions in Northern and Southern Europe Gowan Crop Protection Ltd, UK BioChem agrar, Germany, Report No. 19 48 BTR 0003 GLP Not published ⇨ Filed under KCP 6.10	N	GWI
KCP 5.2	Coleman, H.	2017	Validation of an analytical method for the determination of zoxamide in body fluid and tissue	N	GWI

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 4.2)			Gowan Crop Protection Ltd., UK Battelle UK Ltd., Essex, UK, Report No. FF/17/002 GLP Not published		
KCP 5.2	Freschi, G.	2001	Validation of analytical method for determination of residues of Cymoxanil in tomato (whole fruit) Oxon Italia S.p.A., Italy Sipcam S.p.A, Italy, Report No. SIP1278 GLP Not Published	N	GWI Sipcam Oxon S.p.A.
KCP 5.2	Freschi, G.	2001	Validation of analytical method for determination of residues of Cymoxanil in grapes (bunches) Oxon Italia S.p.A., Italy Sipcam S.p.A, Italy, Report No. SIP1276 GLP Not Published	N	GWI Sipcam Oxon S.p.A.
KCP 5.2	Leak, T.	2010	Analytical method for the determination of Cymoxanil and IN-KQ960 in water (pond, stream, well and tap) using LC/MS/MS Oxon Italia S.p.A., Italy ChemService, Italy, Report No. DuPont-27500/ ABC-65072 rev.2, GLP, Not Published	N	GWI Sipcam Oxon S.p.A.
KCP 5.2	Cermak, J.	2013	Independent Laboratory Validation for the Determination of residues of Cymoxanil and IN-KQ960 in water (drinking and stream) using LC/MS/MS Oxon Italia S.p.A., Italy Research Institute for Organic Syntheses, Inc., Czech Republic, Report No. 2556-256/12/48 (DuPont-35792), GLP, Not published	N	GWI Sipcam Oxon S.p.A.

SIPCAM Oxon S.p.A. is the legal successor of Oxon Italia S.p.A.; Gowan Crop Protection (GWI) is the legal eternity of the company Gowan in Europe

~~Green shaded~~ studies indicated within the Appendix 2 = confirmatory-like studies which are under evaluation by the RMS for Zoxamide in an interzonal procedure.

Grey shaded = data / reference already provided during product authorisation

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

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	Weber, H.	2012	Validation of an enforcement method ("QuEChERS") for the determination of residues of zoxamide in grapes and potatoes and their processed products using LC-MS/MS Eurofins AgroScience Services Chem GmbH, Report No. S12-03949, November 30, 2012 GLP Not published	N	GWI
KCA 4.1.2	Weber, H., Giesau, A.	2013	Validation of analytical method for the determination of residues of the zoxamide metabolite RH-150721 in grapes and processing fractions using LC-MS/MS Eurofins AgroScience Services Chem GmbH, Report No. S12-03950 GLP Not published	N	GWI
KCA 4.1.2	Weber, H., Giesau, A.	2013	Validation of analytical method for the determination of residues of the zoxamide metabolites RH-1452 and RH- 1455 in potatoes and processing fractions using LCMS/MS Eurofins AgroScience Services Chem GmbH, Report No. S12-03951, May 2,02013 GLP Not published	N	GWI
KCA 4.1.2	Luciani, G.P.	2010	Determination of zoxamide residues after five applications of ELECTIS MZ and ZOXIUM 240 SC on potato under field conditions – Italian trial Agri Paradigma S.r.l., Report No. AGRI 012/10 GLP DEC, October 29 2010 GLP Not published	N	GWI
KCA 4.1.2	Nixon, W.B., Sulairman, M.W.	1997	The analysis of RH-117,281 technical in filtered saltwater in support Rohm and Haas, Report No. 95RC-0275, May 15, 1999 Wildlife International Ltd, Report No. 129A-136 GLP Not published	N	GWI
KCA 4.1.2	Kendall, T. Z.	1998	The analysis of RH-117,281 technical in filtered saltwater Rohm and Haas, Report No. 97RC-0077, June 17, 1998 Wildlife International Ltd, Report No. 129A-142	N	GWI

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
			GLP Not published		
KCA 4.1.2	xxx	2010	Validation of an analytical method for the determination of zoxamide in solutions of aquatic toxicity test with GOW 008 xxx, Report No. BT102/10, November 18, 2010 GLP Not published	N	GWI
KCA 4.1.2	Weber, H., Zetzsch, A., Giesler, W.	2016	Storage Stability of residues of zoxamide, RH-150721, RH-1452 and RH-1455 in grape and processed products and potato Eurofins AgroScience Services Chem GmbH, Report No. S12-03952, February 15, 2016 GLP Not published	N	GWI
KCA 4.1.2	Luciani, G.P.	2010	Determination of zoxamide residues after five application of ELECTIS MZ and ZOXIUM 240 SC on Wine grape and Table grape – Italian trial, year 2010 Research Centre “Agri Paradigma S.r.l”, Report No. AGRI 010/10 GLP DEC, October 29, 2010 GLP Not published	N	GWI
KCA 4.1.2	Wais, A.	2001	Determination of residues of RH-117,281 and mancozeb in/on vine grapes (RAC grapes and processing products) following treatment with RH-7281/mancozeb 75WG from a field trial (semi residue decline study) in Italy; 1999 Rohm and Haas, Report No. ER ref R 77.10 RCC Ltd., Report No. 734580 GLP Not published	N	GWI
KCA 4.1.2	Hein, W.	2014	Extraction efficiency of [phenyl-UL-14C] zoxamide from plant metabolism samples (Pea) RLP AgroScience GmbH, Report No. AS362, June 25, 2014 GLP Not published	N	GWI

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 4.2	Weber, H.	2012	Validation of an enforcement method (“QuEChERS”) for the determination of residues of zoxamide in grapes and potatoes and their processed products using LC-MS/MS Eurofins AgroScience Services Chem GmbH, Report No. S12-03949, November 30, 2012 GLP Not published	N	GWI
KCA 4.2	Richter, S.	2014	Validation of the QuEChERS multi-residue method for the determination of zoxamide in various crop types PTRL Europe, Report No. P 3114G, February, 2010 GLP Not published	N	GWI
KCA 4.2	Schlewitz, P.	2014	Independent laboratory validation of the analytical method for the determination of zoxamide residues in lettuce Anadiag, Report No. R B4023, May, 23/2014 GLP Not published	N	GWI
KCA 4.2	Jooß, S.	2013	Development and validation of a residue method for the determination of zoxamide in soil PTRL Europe, Report No. P 3051 G, October 21, 2013 GLP Not published	N	GWI
KCA 4.2	Jooß, S.	2013	Development and validation of a residue method for the determination of zoxamide in drinking and in surface water PTRL Europe, Report No. P 3050 G GLP Not published	N	GWI
KCA 4.2	Schlewitz, P.	2014	Independent laboratory validation of a residue method for the determination of zoxamide in drinking water Anadiag, Report No. R B4049, July 1, 2014 GLP Not published	N	GWI

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 4.2	Miller, C.	2014	Zoxamide: Validation of methodology for the determination of residues in air Huntingdon Life Sciences, Report No. FRK0048, January 29, 2014 GLP Not published	N	GWI
KCA 4.2	Cermak, J.	2013	Independent laboratory validation for the determination of residues of cymoxanil and IN-KQ960 in water (drinking and stream) using LC/MS/MS Výzkumný ústav organických syntéz a.s. (Research Institute for Organic Syntheses, Inc.) Centre of Ecology, Toxicology and Analytics Rybitvi 296, 533 54 Rybitvi, Czech Republic Report no. 255-256/12/48 (DuPont-35792) GLP, Not published	N	Sipcam Oxon S.p.A.* Du Pont
KCA 4.2	Leak, T.	2013	Analytical method for the determination of cymoxanil and IN-KQ960 in water (pond, stream, well and tap) using LC/MS/MS ABC Laboratories, Inc. 7200 East ABC Lane. Columbia, MO 65202 Report no. DuPont-27500 rev. 2/ABC-65072 GLP, Not published	N	Sipcam Oxon S.p.A.* Du Pont
KCA 4.2	Freschi, G.	2001b	Validation of the analytical method for determination of residues of cymoxanil in potato (tuber) SIPCAM S.p.A, Salerano sul Lambro, Italy SIP1277 GLP: Yes Published: No	N	Sipcam Oxon S.p.A.*
KCA 4.2	Wasser, C.	2002	Validation of analytical method for determination of residues of cymoxanil in specimens of tomato, grapes, potatoes and lettuce Anadiag S.A., Haguenau, France A0087 GLP: Yes Published: No	N	Sipcam Oxon S.p.A.*

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 4.2	Freschi, G.	2001a	Validation of the analytical method for determination of residues of cymoxanil in lettuces (plant) SIPCAM S.p.A, Salerano sul Lambro, Italy SIP1279 GLP: Yes Published: No	N	Sipcam Oxon S.p.A.*
KCA 4.2	Melkebeke, T.	2000a	Validation of an analytical method for the determination of cymoxanil residues in soil Notox B.V., 's-Hertogenbosch, The Netherlands 281802 GLP: Yes Published: No	N	Sipcam Oxon S.p.A.*
KCA 4.2	Cabusas, M.E.Y.	1999	Analytical method for the determination of cymoxanil in drinking, ground, and surface water using liquid chromatography with ultraviolet detection DuPont Experimental Station DuPont-2126 GLP: No Published: No	N	Sipcam Oxon S.p.A.*
KCA 4.2	Melkebeke, T.	2000b	Validation of an analytical method for the determination of cymoxanil in air. Notox B.V., 's-Hertogenbosch, The Netherlands 257805 GLP: No Published: No	N	Sipcam Oxon S.p.A.*

GWI = Gowan Company
*: *formerly Oxon Italia S.p.A*

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

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 4.2	Burdge, E., Kurilla, K., Guo, I., Hofmann, C.	1999	Tolerance Enforcement Method for Parent RH-117,281 in Tomato RAC and Processed Fractions Rohm and Haas Company, USA Enviro-Test Laboratories (ETL), Canada, Report No. 34-99-111, March 3, 1999 GLP Not published	N	GWI
KCA 4.2	Bruns, G., Gottschalk, R.	1999	Independent Laboratory Validation (ILV) Trials of Tolerance Enforcement Method for Parent RH-117,281 in tomato RAC and Processed Fractions (TR 34-99-111) Rohm and Haas Company, USA Enviro-Test Laboratories (ETL), Canada, Report No. 34-99-188, December 15, 1999 GLP Not published	N	GWI

GWI = Gowan Company

The following tables are to be completed by MS

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 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 zoxamide

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

A 2.1.1.1 Description of analytical methods for the determination of residues in plants

A 2.1.1.1.1 Analytical method 1 – determination of zoxamide and its metabolites in grape fruits and processed commodities (BPL-STUDY-18-000085)

The analytical phase of the following studies was performed according to method BPL-STUDY-18-000085. The method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4, for the determination of zoxamide (as sum of R and S isomers), (R)-zoxamide, (S)-zoxamide, metabolites RH-141452, RH-150721 (as sum of R and S isomers), (R)-RH-150721, (S)-RH-150721, RH-129151 (as sum of R and S isomers), (R)-RH-129151, (S)- RH-129151, RH-24549, RH-141288 (as sum of R and S isomers), (R)-RH-141288 and (S)-RH-141288 in Raw Agricultural Commodity grapes (bunches) and/or processed samples (raisins) and is presented under KCP 5.2 as post-authorisation method. The analytical method principle is based on the QuEChERS-method using HPLC-MS/MS.

During the course of the following supervised residue trials procedural recoveries were performed and are presented.

A 2.1.1.1.1.1 Method validation

Comments of zRMS:	The study is accepted. 2 grapes residue trials were conducted in NEU. The objective of the study was to determine the magnitude of residues of zoxamide (R & S and sum) and its metabolites [RH-150721 (R & S), RH-141288 (R & S), RH-129151 (A and B), RH-24549 and RH-141452] in raw agricultural commodity specimens of grapevine (RAC bunches) and processed fractions after five applications of Zoxium 240 SC. Target application rate was 0.75 L/ha and target application timing was 60 (± 4), 52 (± 4), 44 (± 3), 36 (± 3) and 28 (± 2) days before harvest. Two deviations were observed during the Analytical Phase of the Study. They were all considered without impact on the study results. The analytical method was described and fully validated according to guidelines SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 in a previous study (LabAnalysis Study BPL-STUDY-18-000085). ~~For evaluation of 5.1/01, 02, 03, 06 studies please, see Section 7 of the present RR. Studies 5.1/04, 05, 07, 08, 09, 10, 11 are zoxamide confirmatory studies currently being evaluated by zoxamide RMS (Latvia).~~ These studies are also for SEU.

Reference:	**KCP 5.1/01**
Report:	Peterek, S., 2020: Magnitude of the residues of zoxamide and its metabolites in grapevine (RAC bunches) and processed fractions, following applications of Zoxium 240 SC, Northern Europe – 2018

	Gowan Crop Protection Ltd., UK Staphyt GmbH, Germany, Report No. AB2-18-35355, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	Due to response instability in analytical sequence with both solvent and matrix standards, the matrix effects were not calculated. This deviation from the study plan is regarded to be not relevant for the integrity of the study since the analysis of residues has been performed by matrix matched standards. Mean recoveries below 70 % were found for RH-129151 A and RH-129151 B in young wine at 0.05 mg/L, while the corresponding mean recoveries at LOQ were in the range 70 - 110 %. However, as the residues of these analytes in young wine samples were found below the LOQ, the results were not corrected by taking into account a recovery factor. For analytes/matrices combination with mean recoveries > 110 %, the high recovery is probably due to the interconversion among the analytes, present at the same time and in the same concentration in the recovery extracts, while in the corresponding "real" samples this condition does not occur. The recovery factor was not applied to the residues results in order to remain the worst-case.
GLP:	Yes
Acceptability:	Yes

and

Comments of zRMS:	The study is acceptable. The analytical determination was carried out using a HPLC-MS/MS method validated according to SANCO/825/00 rev.8.1 and SANCO/3029/99 rev. 4 guidelines.

Reference:	**KCP 5.1/02**
Report	Sala, A., 2020: Determination of zoxamide and its metabolites in raw agricultural commodity wine grape (berries) and processed fractions (juice, wine) following five applications of Zoxium 240 SC (GWN-9790 EU) in open field condition, 2 harvest trials, Northern Europe, year 2017 - final report amendment no. 1 Gowan Crop Protection Ltd, UK Lab Analysis, Italy, Report No. BPL-STUDY-19-000041, GLP, Not published

and

Comments of zRMS:	The study is acceptable. The analytical determination was carried out using a HPLC-MS/MS method validated according to SANCO/825/00 rev.8.1 and SANCO/3029/99 rev. 4 guidelines.

Reference:	**KCP 5.1/03**
Report	Thomas-Delille, E., 2020: Determination of zoxamide and its metabolite RH-

	150721 residues in wine grape and processed fractions following five foliar applications with Zoxium 240 SC under field conditions in Northern Europe in 2017 – amended final report Gowan Crop Protection Ltd, UK Anadiag, France, Report No. B7284, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	Deviation on trial B7284 BW1: Due to the mechanically harvest of the vineyard by the farmer, the field principal investigator anticipated the sampling at harvest and performed is 20 days after last application instead of 28 (±2) days after last application, as required by the study plan. Deviation on trial B7284 BW1: At sampling the minimum weight of 60 kg required for the specimens B7284 BW1/UH/P and B7284 BW1/TH/P was not reached; it was only 58.438 kg and 58.091 kg, respectively. This deviation has no impact on the integrity of the data since the weights were sufficient to perform the processing phase. Deviation on trial B7284 CZ1: The samples for processing were shipped under ambient conditions instead of under refrigerated conditions as required by the study plan due to organisational reason. However, the sanitary status of the specimens was good at receipt. The processing phase of trial B7284 BW1 was started 14 days after receipt of the samples instead of the next day after receipt at the samples, as required by the study plan. However, according to the analytical results, this deviation was regarded acceptable.
GLP:	Yes
Acceptability:	Partially

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/04**
Report	Sala, A., 2020: Determination of zoxamide and its metabolites in raw agri-cultural commodity wine grape (berries) and processed fractions (juice, wine) following five applications of Zoxium 240 SC (GWN-9790 EU) in open field condition 2 harvest trials, Southern Europe, year 2017 - final report amendment no. 1 Gowan Crop Protection Limited, UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000051, GLP, Not published

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/05**
Report	Casalinouvo, L., 2020: Determination of zoxamide and his metabolite RH-150721 residues raw agricultural commodity red grapes and processed fraction following five applications of Zoxium 240 SC (zoxamide 240 g/L) (South Europe – 2 trials year 2017) - plus amendment no. 1 to final report Gowan Crop Protection Limited, UK BioSphereS by Biotecnologie B.T., Italy, Report No. BIU-005-17, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	Due to the dry season the bunches did not reach the quantity assumed by the study plan. As a result, from trial I/ZO17/GR01 only 26.5 and 24 kg were collected from the control and the treated plot, respectively, and from trial I/ZO17/GR02 only 20 and 22 kg from the control and the treated plot, respectively. However, these amounts were regarded sufficient for the intended processing phase and thus the study plan deviation regarded to not impact the integrity of the study. Due to the limited bunch weights the juice samples received from trial I/ZO17/GR02 were also reduced, but still sufficient for the following residue analysis. This deviation was therefore regarded to not impact the integrity of the study. The following recovery checks were slightly higher than the intended accuracy validation range (70-110%): 19/51/GJ/RC3/NH/2: 118.9% for analyte (S)-zoxamide 19/51/GJ/RC1/NH: 118.8% for analyte (S)-RH-141288 19/51/GJ/RC2/NH: 113.6% for analyte (S)-RH-141288 19/51/WI/RC1/NH: 121.7% for analyte (R)-RH-150721 19/51/WI/RC2/NH: 118.5% for analyte (R)-RH-150721 19/51/WI/RC1/NH: 114.8% for analyte (S)-RH-150721 19/51/WI/RC2/NH: 124.4% for analyte (S)-RH-150721 However, these deviations were regarded to have no impact on the integrity of the study results since the recoveries reported above are slightly higher than maximum allowed (70-110%) – and therefore represent worst-case values.
GLP:	Yes
Acceptability:	Yes

and

Comments of zRMS:	The study is acceptable. The objective of this study was the determination of the residues of its enantiomers (R)-Zoxamide and (S)-Zoxamide (an the sum), and of the following Zoxamide metabolites: RH-150721 (as sum of R and S enantiomers) and its enantiomers (R)-RH-150721, (S)-RH-150721; RH-129151 (as sum of R and S enantiomers) and its enantiomers (R)-RH-129151 and (S)-RH-129151; RH-141288 (as sum of R and S enantiomers) and its enantiomers (R)-RH-141288 and (S)-RH-141288; RH-24549 and RH-141452 in grape wine, coming from 1 harvest trial and 3 decline trials per-

	formed in open field in NEU. Each trial was carried out performing 5 and 3 applications of the product ZOXIUM 240 SC (GWN 9790 EU). The analytical method was validated according to the SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 guidelines in the BPL-STUDY-18-000085.

Reference:	**KCP 5.1/06**
Report	Longhi, D., 2020: Determination of zoxamide and its metabolites in raw agricultural commodity of grape wine in open field following five and three applications of the formulated product GWN-9790 EU (North Europe - 4 trials year 2019) Gowan Crop Protection Ltd, UK Lab Analysis, Italy, Report No. BPL-STUDY-19-000057, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	In the field trial CMN-19-38746-FR01 on the T1 plot at applications A4 and A5 a deviation from the target dose of GWN 9790 EU was noted at +10.5%, being slightly above the intended +/- 10% because of a fluctuation of the sprayer flow rate. For correction, the correct amount of the applied test item dose has been recalculated. This deviation has been regarded to not influence the integrity of the study results. In the field trials CMN-19-38746-HU03 and CMN-19-38746-FR02 the T1 plots were sampled before the T2 plots though it should have been inversely, as T1 plots were treated less times. This deviation has been regarded to not influence the integrity of the study results. In the field trial CMN-19-38746-HU03 the application no. 4 was done 10 days after the application no. 3. However, this is still within the ±25 % range of an intended application interval of 8 days. Besides, other applications were spaced by 7 days, balancing the interval to a target range of nominally 7-8 (+1) days.
GLP:	Yes
Acceptability:	Yes

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/07**
Report	Longhi, D., 2020: Determination of zoxamide and its metabolites in raw agricultural commodity of table grape and processed (raisin) in open field following five and three applications of the formulated product GWN 9790 EU (South Europe – 1 trial year 2019) Gowan Crop Protection Ltd., UK LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-19-000058, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010)

	SANCO/3029/99 rev. 4 (2000)
Deviations:	The recovery values for the analytes (R)-RH-150721 and (S)-RH-150721 on recovery check samples BPL-SMPL-19-002114/NH RC1 and BPL-SMPL-19-002114/NH RC2 were found to be above the range allowed by the SANCO/3030/99 rev. 4 and SANCO/825/00 rev. 8.1 (70 - 110%). Since the recovery values were above the permitted range, the concentrations of the analytes under examination have been overestimated, what is regarded a worst-case. Since the analytes (R)-RH-150721 and (S)-RH-150721 were still detected at concentrations <LOQ, the overestimation was regarded to have no impact on the integrity of the results.
GLP:	Yes
Acceptability:	Yes

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/08**
Report:	Maccaferri, L., 2020: Magnitude of the residues of zoxamide in table grape bunches and in raisins processed fraction, following applications of Zoxium 240 SC. One harvest trial, Southern Europe – 2018 Gowan Crop Protection Ltd., UK Renolab S.r.l. Italy, Report No. 18097-03R, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviation(s):	Due to response instability in analytical sequence with both solvent and matrix standards, the matrix effects were not calculated. This deviation was regarded as not relevant for the integrity of the study since the analysis is performed with matrix matched standards. For some analyte / matrix combinations the mean recovery was >110 % but <120 %. Since recovery values of 70-120% for the here implied concentration ranges of 0.01-0.1 mg/kg are acceptable according to SANCO/825/00 rev. 8.1 (2010), this deviation from the study plan is regarded as not relevant.
GLP:	Yes
Acceptability:	Yes

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/09**
Report:	Maccaferri, L. 2019: Determination of the residues of zoxamide and/or phosphorous acid in table grape raw agricultural commodity following five applications

of GOW F 716, Zoxium 240 SC, GOW F 316 in open field conditions (one harvest trial, Italy 2017)
Gowan Crop Protection Ltd., UK
Renolab S.r.l., Italy, Report No. 17120-01R, GLP, Not published

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference: **KCP 5.1/10**

Report: Maccaferri, L. 2019: Determination of the residues of zoxamide and/or phosphorous acid in raw agricultural commodity of grapevine and processed commodities (juice, must, young wine and bottled wine) following five applications of GOW F 716, Zoxium 240 SC, GOW F 316 in open field conditions (one harvest trial, Italy 2017)
Gowan Crop Protection Ltd. UK,
Renolab S.r.l., Italy, Report No. 17120-02R, GLP, Not published

and

Comments of zRMS:	The study is not relevant (in the context of residues). Submitted as support for B7 and for SEU – therefore not evaluated.

Reference: **KCA 5.1/11**

Report: Maccaferri, L., 2020: Magnitude of the residues of zoxamide enantiomers and metabolites in grapes and processed commodities (juice, must, young wine and bottled wine) following five applications of GOW 716 and Zoxium 240 SC in open field condition (Italy 2017)
Gowan Crop Protection Ltd., UK
Renolab S.r.l. Italy, Report No. 19200-01R, GLP, Not published

Guideline(s): SANCO/825/00 rev. 8.1 (2010)
SANCO/3029/99 rev. 4 (2000)

Deviation(s): Due to response instability in analytical sequence with both solvent and matrix standards, the matrix effects were not calculated. This deviation was regarded as not relevant for the integrity of the study since the analysis is performed with matrix matched standards.
For some analyte / matrix combinations the mean recovery was >110 % but ≤120 %. Since recovery values of 70-120% for the here implied concentration ranges of 0.01-0.1 mg/kg and below (0.005-0.05 mg/kg) are acceptable according to SANCO/825/00 rev. 8.1 (2010), this deviation from the study plan is regarded as not relevant.

For the following analyte / matrix combination the mean recovery was < 70 %: (A) RH-129151 and (B) RH-129151 at 0.05 mg/L in young wine. However, the

	overall mean recovery for young wine (at different fortification levels) was 72.7 ±15.9 %.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical phase of the studies was performed according to method BPL-STUDY-18-000085. The method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4, for the determination of zoxamide (as sum of R and S isomers), (R)-zoxamide, (S)-zoxamide, metabolites RH-141452, RH-150721 (as sum of R and S isomers), (R)-RH-150721, (S)-RH-150721, RH-129151 (as sum of R and S isomers), (R)-RH-129151, (S)- RH-129151, RH-24549, RH-141288 (as sum of R and S isomers), (R)-RH-141288 and (S)-RH-141288 in Raw Agricultural Commodity (RAC) grapes (bunches) and/or processed samples (raisins). The analysis was carried out using a HPLC-MS/MS method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 in the study report SALA (2020), BPL-STUDY-18-000085: "Validation of an analytical method to determine zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities". The method is presented under KCP 5.2 as post-authorisation method. During the course of the supervised residue trials procedural recoveries were performed and are presented.

Aliquots of the samples were weighed and extracted twice with an extraction mixture of water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 extracted fractions were pooled and brought up to a final volume of 40 mL (20 mL for potato flakes and potato chips) using the same solvent mixture. After centrifugation, the extracts were transferred into 2 mL glass HPLC vials for final determination with HPLC-MS techniques.

Since the metabolite RH-141452 is known to form conjugates with matrix components, an additional alkaline hydrolysis step was carried out to break the conjugates and release them in their free forms in order to determine the total amount of the analytes (in addition to their already free amounts available in the commodities). For this purpose, a second sample aliquot was treated with an adequate amount of a mixture of acetonitrile/NaOH and hydrolysed at 40°C for 30'. The sample pellet, after neutralization and removal of the liquid phase, was extracted a second time with water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 liquid phases were pooled and brought up to a final volume. They were measured with the analytical method detailed in study BPL-STUDY-18-000085.

For metabolite RH-129151 the correlation between the absolute configuration of the enantiomer (R) or (S) and the corresponding chromatographic peaks was not available; therefore, the first eluted peak was assigned as RH-129151 (A) and the second eluted peak as RH-129151 (B).

Three chromatographic methods were used:

- Method A, for zoxamide and metabolites RH-150721 and RH-141288, by chiral chromatography
- Method B, for metabolite RH-129151, by chiral chromatography
- Method C, for metabolites RH-141452 and RH-24549, by not chiral chromatography

By methods A and B the fraction of the two enantiomers R and S in the found residues were determined.

The analytes were determined with instrumental methods A, B and C, detailed in the separate study summary of BPL-STUDY-18-000085.

Results and discussions

During the course of the supervised residue trials procedural recoveries were measured as follows :

Table A 1: Results of the recoveries in grapes (AB2-18-35355, 18097-01R)

Analyte	Matrix	Fortification level (mg/kg or mg/L)	Recoveries			Overall mean recovery ± RSD %
			Single values (%)	Mean (%)	RSD (%)	
qualifier transition						
(R)-Zoxamide						
(R)-Zoxamide	Grapevine bunches	0.0050	109.2, 111.3, 112.1, 110.0, 94.7, 88.8	104.3	9.6	99.6 ± 8.4
		0.050	102.9, 101.8, 108.2, 98.9, 97.2	101.8	4.2	
		1.5	94.5, 91.4, 93.4, 90.6, 88.7	91.8	2.5	
	Juice pre-pasteurisation	0.0050	103.6, 105.0, 104.3, 102.1, 104.4	103.9	1.1	100.3 ± 3.9
		0.050	97.3, 96.8, 94.6, 97.0, 98.0	96.7	1.3	
	Juice post-pasteurisation	0.0050	106.1, 104.0, 103.7, 106.1, 103.6	104.7	1.2	101.5 ± 3.7
		0.050	100.4, 97.7, 98.3, 99.6, 95.4	98.3	2.0	
	Must	0.0050	97.9, 104.7, 103.7, 105.1, 104.2	103.1	2.9	98.5 ± 6.7
		0.050	95.4, 90.1, 85.5, 93.4, 88.5	90.6	4.3	
		0.6	103.1, 104.4, 96.4, 102.1, 102.6	101.7	3.1	
	Young wine	0.0050	101.6, 98.1, 104.5, 103.1, 113.6	104.2	5.5	98.1 ± 5.9
		0.050	97.3, 92.2, 93.9, 90.3, 97.8	94.3	3.4	
		0.20	94.1, 97.9, 95.5, 93.8, 98.6	96.0	2.3	
	Bottled wine	0.0050	109.7, 116.0, 108.7, 106.9, 118.2	111.9	4.4	103.6 ± 7.1
		0.050	94.5, 103.7, 97.2, 96.1, 105.0	99.3	4.7	
		0.20	95.6, 96.9, 101.1, 100.2, 103.7	99.5	3.3	
(S)-Zoxamide						
(S)-Zoxamide	Grape bunches	0.0050	112.9, 116.2, 112.3, 105.9, 103.6, 89.4	106.7	9.1	99.9 ± 8.9
		0.050	103.1, 96.5, 104.8, 97.7, 98.7	100.2	3.6	
		1.5	91.6, 93.9, 93.6, 89.4, 88.9	91.5	2.5	
	Juice pre-pasteurisation	0.0050	102.2, 101.8, 102.1, 107.2, 105.3	103.7	2.3	100.4 ± 4.0
		0.050	98.2, 96.2, 94.8, 96.9, 99.5	97.1	1.9	
	Juice post-pasteurisation	0.0050	107.8, 101.3, 100.3, 105.7, 105.6	104.1	3.1	100.9 ± 4.2
		0.050	99.7, 98.1, 96.6, 99.3, 94.6	97.7	2.1	
	Must	0.0050	99.4, 107.8, 103.7, 103.5, 104.6	103.8	2.9	99.4 ± 6.7
		0.050	95.8, 89.6, 86.5, 96.2, 89.0	91.4	4.7	
		0.6	105.4, 104.5, 97.6, 103.5, 103.7	102.9	3.0	
	Young wine	0.0050	105.5, 99.5, 99.3, 103.2, 111.0	103.7	4.7	97.3 ± 5.9
		0.050	95.7, 90.5, 95.7, 89.2, 96.3	93.5	3.6	
		0.20	91.9, 95.3, 95.4, 93.5, 97.2	94.7	2.1	
	Bottled wine	0.0050	115.5, 113.2, 107.1, 107.0, 114.5	111.5	3.7	103.9 ± 6.3
		0.050	96.7, 103.5, 100.8, 96.1, 105.9	100.6	4.2	
		0.20	96.4, 97.7, 100.9, 100.6, 102.8	99.7	2.6	
(R)- RH-150721						

(R)-RH-150721	Grapevine bunches	0.0050	106.3, 83.7, 84.8, 79.9, 84.1, 95.9	89.1	11.2	92.8 ± 9.7
		0.050	98.3, 89.3, 105.7, 96.9, 95.5	97.1	6.1	
	Juice pre-pasteurisation	0.0050	100.6, 94.9, 99.8, 102.4, 102.4	100.0	3.1	93.8 ± 7.7
		0.050	90.4, 85.2, 85.9, 84.9, 91.1	87.5	3.4	
	Juice post-pasteurisation	0.0050	101.9, 100.2, 95.6, 97.6, 100.8	99.2	2.6	95.9 ± 4.6
		0.050	96.9, 94.4, 88.6, 93.3, 90.1	92.7	3.6	
	Must	0.0050	101.5, 98.7, 97.2, 90.8, 96.7	97.0	4.1	93.0 ± 7.7
		0.050	100.8, 89.2, 83.7, 90.8, 80.8	89.0	8.6	
	Young wine	0.0050	79.0, 92.3, 94.3, 94.3, 101.7	92.3	8.9	89.8 ± 7.2
		0.050	89.7, 86.1, 85.3, 83.6, 91.3	87.2	3.7	
	Bottled Wine	0.0050	107.8, 112.5, 103.8, 102.5, 96.2	104.6	5.8	98.8 ± 7.7
		0.050	90.6, 96.8, 95.5, 92.3, 90.1	93.1	3.2	
(S)-RH-150721						
(S)-RH-150721	Grape bunches	0.0050	102.8, 71.2, 79.4, 84.6, 74.6, 99.0	85.3	15.2	90.3 ± 13.1
		0.050	98.0, 86.8, 107.4, 95.0, 94.0	96.2	7.8	
	Juice pre-pasteurisation	0.0050	96.9, 99.9, 97.8, 98.5, 94.9	97.6	1.9	92.2 ± 6.6
		0.050	88.6, 88.4, 85.5, 83.2, 88.2	86.8	2.7	
	Juice post-pasteurisation	0.0050	102.2, 99.5, 103.2, 102.1, 101.9	101.8	1.4	96.8 ± 5.8
		0.050	94.5, 92.3, 90.4, 93.4, 88.2	91.8	2.7	
	Must	0.0050	101.3, 104.3, 102.4, 99.4, 88.1	99.1	6.5	93.9 ± 9.7
		0.050	103.4, 87.9, 82.9, 88.6, 80.9	88.7	10.0	
	Young wine	0.0050	100.8, 88.1, 98.1, 99.6, 100.4	97.4	5.5	91.0 ± 9.4
		0.050	94.6, 78.3, 83.2, 83.5, 83.8	84.7	7.0	
	Bottled Wine	0.0050	104.3, 109.6, 100.8, 99.4, 92.7	101.4	6.2	97.5 ± 6.2
		0.050	93.7, 92.9, 95.8, 95.1, 90.4	93.6	2.2	
(R)-RH-141288						
(R)-RH-141288	Grapevine bunches	0.0050	104.3, 111.0, 116.7, 95.0, 100.1, 84.6	101.9	11.2	100.1 ± 8.9
		0.050	105.5, 96.2, 92.1, 96.5, 98.9	97.8	5.0	
	Juice pre-pasteurisation	0.0050	106.3, 103.3, 104.5, 101.7, 103.6	103.9	1.6	99.4 ± 5.3
		0.050	96.6, 94.6, 90.1, 94.7, 98.8	94.9	3.4	
	Juice post-pasteurisation	0.0050	105.1, 104.7, 103.7, 108.5, 98.5	104.1	3.5	100.7 ± 4.6
		0.050	100.2, 98.4, 94.7, 98.4, 94.4	97.2	2.6	
	Must	0.0050	102.7, 104.1, 104.3, 102.3, 98.2	102.3	2.4	96.3 ± 7.4
		0.050	96.7, 88.5, 87.6, 91.8, 86.6	90.3	4.6	
	Young wine	0.0050	88.2, 96.5, 94.2, 97.3, 93.8	94.0	3.8	90.7 ± 4.8
		0.050	86.8, 89.4, 87.5, 86.8, 86.2	87.4	1.4	
	Bottled Wine	0.0050	109.2, 112.1, 106.1, 106.6, 118.1	110.4	4.4	104.4 ± 8.0
		0.050	100.3, 94.8, 93.8, 108.8	98.3	6.6	
(S)-RH-141288						

(S)-RH-141288	Grapevine bunches	0.0050	116.0, 120.2, 112.2, 101.5, 104.2, 87.2	106.9	11.2	105.2 ± 9.9
		0.050	100.2, 96.3, 116.9, 107.6, 94.7	103.2	8.9	
	Juice pre-pasteurisation	0.0050	107.1, 100.3, 102.1, 104.0, 106.5	104.0	2.8	99.3 ± 6.1
		0.050	100.0, 95.3, 88.3, 94.1, 95.2	94.6	4.4	
	Juice post-pasteurisation	0.0050	105.3, 99.0, 101.4, 99.5, 104.4	101.9	2.8	100.2 ± 2.9
		0.050	100.5, 98.1, 97.8, 99.8, 95.8	98.4	1.9	
	Must	0.0050	99.3, 104.4, 105.1, 97.7, 103.9	102.1	3.3	95.3 ± 8.5
		0.050	94.8, 88.1, 85.3, 90.8, 83.5	88.5	5.1	
	Young wine	0.0050	98.0, 105.9, 104.5, 100.4, 104.1	102.6	3.2	98.6 ± 5.0
		0.050	93.3, 93.0, 96.2, 92.9, 98.2	94.7	2.5	
	Bottled Wine	0.0050	105.2, 120.6, 109.2, 105.2, 118.0	111.6	6.5	105.4 ± 8.3
		0.050	92.9, 103.9, 99.0, 95.8, 104.2	99.2	5.0	
(A)-RH-129151						
(A)-RH-129151	Grapevine bunches	0.0050	120.0, 104.8, 110.4, 81.4, 87.7	100.9	15.9	100.0 ± 15.5
		0.050	93.2, 122.9, 76.6, 102.5, 100.4	99.1	16.9	
	Juice pre-pasteurisation	0.0050	100.8, 73.9, 106.2, 105.4, 114.8	100.2	15.5	93.7 ± 17.2
		0.050	105.5, 63.0, 85.6, 90.2, 91.6	87.2	17.7	
	Juice post-pasteurisation	0.0050	119.7, 117.1, 111.1, 103.8, 125.6	115.5	7.2	109.2 ± 10.2
		0.050	113.7, 103.4, 86.1, 101.2, 110.7	103.0	10.5	
	Must	0.0050	106.6, 106.0, 105.3, 103.7, 99.9	104.3	2.6	101.3 ± 5.3
		0.050	104.5, 101.9, 89.2, 99.8, 96.3	98.3	6.0	
	Young wine	0.0050	72.5, 77.3, 64.2, 79.4, 100.8	78.8	17.3	70.9 ± 18.3
		0.050	60.5, 70.4, 63.8, 54.7, 65.2	62.9	9.2	
	Bottled Wine	0.0050	123.1, 113.3, 116.2, 115.6, 133.3	120.3	6.8	113.7 ± 8.9
		0.050	103.7, 102.5, 119.7, 102.0, 107.9	107.1	6.9	
(B)-RH-129151						
(B)-RH-129151	Grapevine bunches	0.0050	112.3, 109.3, 103.2, 77.7, 86.0	97.7	15.5	98.6 ± 15.3
		0.050	91.2, 121.1, 76.8, 108.3, 99.8	99.4	16.9	
	Juice pre-pasteurisation	0.0050	105.3, 96.4, 103.3, 106.0, 110.3	104.3	4.9	96.4 ± 10.3
		0.050	98.1, 87.6, 80.1, 86.1, 91.4	88.9	7.5	
	Juice post-pasteurisation	0.0050	118.6, 116.9, 114.5, 105.4, 128.9	116.9	7.2	110.3 ± 10.2
		0.050	114.0, 100.7, 87.6, 106.7, 109.2	103.6	9.8	
	Must	0.0050	100.4, 104.2, 102.8, 98.8, 102.3	101.7	2.1	100.6 ± 4.3
		0.050	105.1, 102.1, 89.9, 102.4, 98.5	99.6	5.9	
	Young wine	0.0050	72.4, 80.5, 63.5, 89.4, 101.8	81.5	18.2	72.0 ± 20.1
		0.050	58.4, 67.2, 66.7, 56.3, 63.9	62.5	7.9	
	Bottled Wine	0.0050	110.7, 105.2, 126.0, 124.4, 132.8	119.8	9.5	114.0 ± 9.8
		0.050	102.2, 101.6, 119.7, 104.1, 113.8	108.3	7.4	
RH-24549						

RH-24549	Grapevine bunches	0.010	111.1, 113.1, 97.5, 104.5, 108.6	106.9	5.8	107.5 ± 6.3
		0.10	115.1, 110.0, 115.5, 102.1, 97.6	108.1	7.4	
	Juice pre-pasteurisation	0.010	93.8, 86.2, 100.1, 106.4, 108.0	98.9	9.2	98.4 ± 6.7
		0.10	95.6, 94.5, 95.7, 102.9, 100.7	97.9	3.8	
	Juice post-pasteurisation	0.010	115.8, 105.3, 99.6, 116.6, 112.8	110.0	6.7	112.3 ± 5.7
		0.10	108.0, 111.9, 117.4, 120.4, 115.4	114.6	4.2	
	Must	0.010	78.2, 84.1, 115.3, 115.1, 118.3	102.2	19.0	106.6 ± 13.1
		0.10	114.7, 105.9, 109.8, 113.8, 110.7	111.0	3.2	
	Young wine	0.010	101.0, 105.5, 118.1, 113.8, 121.3	112.0	7.6	110.9 ± 6.1
		0.10	103.2, 108.9, 107.4, 112.4, 117.3	109.8	4.8	
	Bottled wine	0.010	108.2, 110.8, 114.8, 124.3, 119.0	115.4	5.6	113.2 ± 5.6
		0.10	103.1, 108.7, 109.3, 115.8, 118.2	111.0	5.4	
Free RH-141452						
Free RH-141452	Grapevine bunches	0.010	104.5, 115.3, 115.5, 113.6, 108.9	111.6	4.3	110.9 ± 5.9
		0.10	116.8, 113.6, 118.7, 101.5, 100.8	110.3	7.7	
	Juice pre-pasteurisation	0.010	99.9, 116.7, 104.6, 116.2, 112.7	110.0	6.8	107.4 ± 5.5
		0.10	103.4, 107.7,.106.0,.104.2,.102.3	104.7	2.0	
	Juice post-pasteurisation	0.010	107.7, 108.3, 103.8, 108.7, 116.5	109.0	4.2	112.2 ± 4.4
		0.10	115.9, 117.5, 113.0, 118.7, 112.2	115.5	2.4	
	Must	0.010	107.0, 102.2, 103.0, 104.7, 108.6	105.1	2.6	106.0 ± 2.6
		0.10	110.0, 108.1, 106.5, 107.9, 102.5	107.0	2.6	
	Young wine	0.010	87.2, 102.8, 109.2, 104.3, 116.9	104.1	10.5	105.7 ± 7.4
		0.10	104.5, 112.9, 106.2, 109.1, 104.0	107.3	3.4	
	Bottled wine	0.010	104.6, 107.2, 103.2, 107.8, 104.8	105.5	1.8	108.4 ± 3.6
		0.10	111.5, 110.9, 111.7, 115.5, 106.8	111.3	2.8	
Total RH-141452						
Total RH-141452	Grapevine bunches	0.010	87.2, 97.9, 91.2, 90.9, 87.9	91.0	4.6	101.5 ± 11.4
		0.10	111.6, 107.7, 115.0, 112.8, 112.6	112.0	2.4	
	Juice pre-pasteurisation	0.010	113.2, 110.3, 100.3, 99.5, 105.3	105.7	5.7	104.6 ± 4.1
		0.10	103.3, 103.7, 105.7, 101.5, 102.8	103.4	1.5	
	Juice post-pasteurisation	0.010	110.7, 112.5, 118.8, 111.8, 118.6	114.5	3.4	111.0 ± 4.4
		0.10	102.8, 108.2, 107.6, 107.7, 110.8	107.4	2.7	
	Must	0.010	114.2, 117.9, 119.3, 111.3, 118.5	116.2	2.9	112.8 ± 4.0
		0.10	109.6, 107.1, 111.3, 107.1, 111.7	109.4	2.0	
	Young wine	0.010	131.5, 119.3, 104.0, 97.0, 115.8	113.5	11.8	110.6 ± 9.3
		0.10	110.4, 111.9, 100.5, 102.0, 113.4	107.7	5.5	
	Bottled wine	0.010	110.9, 101.5, 109.0, 109.0, 108.3	107.7	3.4	106.5 ± 2.8
		0.10	103.6, 107.1, 106.3, 103.6, 105.8	105.3	1.5	

For the following analytes and matrices in the study AB2-18-35355 the mean recovery was > 110 %:

- (S)-zoxamide, (R)-RH 141288 and (S)-RH-141288 at 0.005 mg/L in bottled wine
- (A) RH-129151 and (B) RH-129151 at 0.005 mg/L in juice post-pasteurisation and bottled wine
- RH-24549 at 0.01 mg/L in juice post-pasteurisation, young wine and bottled wine; at 0.1 mg/L in juice post-pasteurisation and bottled wine
- Free RH-141452 at 0.1 mg/L in juice post-pasteurisation
- Total RH-141452: at 0.01 mg/Kg-L in juice post-pasteurisation, in must and in young wine; at 0.1 mg/kg-L in grape, in juice post-pasteurisation and in young wine

For the following analytes and matrices, the mean recovery was < 70 %:

- (A) RH-129151 and (B) RH-129151 at 0.05 mg/L in young wine.

Table A 2: Recovery results in grapes and processed commodities (BPL-STUDY-19-000041)

Analyte	Recovery (%)	
	LOQ	10xLOQ
Grape berries		
Not hydrolysed samples		
(R)-Zoxamide	108.5	88.0
(S)-Zoxamide	106.8	93.6
(R)-RH-150721	102.6	93.1
(S)-RH-150721	91.9	98.2
(R)-RH-141288	91.7	100.0
(S)-RH-141288	100.4	94.1
RH-129151 (A)	106.5	72.7
RH-129151 (B)	100.6	76.1
RH-24549	83.8	101.6
RH-141452	92.3	108.7
Hydrolysed samples		
RH-141452	92.3	89.9
Grape juice		
Not hydrolysed samples		
(R)-Zoxamide	95.6	92.7
(S)-Zoxamide	95.1	92.5
(R)-RH-150721	96.9	98.5
(S)-RH-150721	84.4	93.9
(R)-RH-141288	80.6	91.8
(S)-RH-141288	89.5	93.3
RH-129151 (A)	105.2	84.10
RH-129151 (B)	106.4	87.9
RH-24549	83.5	96.1
RH-141452	75.2	99.0

Hydrolysed samples		
RH-141452	102.3	104.9
Wine		
Not hydrolysed samples		
(R)-Zoxamide	85.0	92.2
(S)-Zoxamide	103.3	91.4
(R)-RH-150721	94.0	95.8
(S)-RH-150721	90.6	94.7
(R)-RH-141288	100.9	91.3
(S)-RH-141288	91.9	92.3
RH-129151 (A)	108.4	87.8
RH-129151 (B)	84.4	83.3
RH-24549	91.3	96.2
RH-141452	95.8	98.5
Hydrolysed samples		
RH-141452	97.1	102.9

All recovery values were between 70-110%.

Table A 3: Recovery results in wine (BPL-STUDY-19-000057)

Analyte	**Recovery (%)**	
	LOQ	**100xLOQ**
Grape wine		
Not hydrolysed samples		
Zoxamide (R)	102.3	109.1
Zoxamide (S)	100.3	104.9
RH-141288 (R)	106.1	103.8
RH-141288 (S)	101.7	103.2
RH-150721 (R)	109.1	102.1
RH-150721 (S)	106.2	104.6
RH-129151 (A)	88.4	89.0
RH-129151 (B)	95.8	80.9
RH-24549	83.52	106.95
RH-141452	86.3	105.52
Hydrolysed samples		
RH-141452	84.59	89.52

All recovery values were between 70-110%.

Table A 4: Recovery results in grapes and processed commodities (BPL-STUDY-19-000051)

Analyte	Recovery (%)	
	LOQ	10xLOQ
Grape berries		
Not hydrolysed samples		
(R)-Zoxamide	98.7	100.7
(S)-Zoxamide	104.3	95.4
(R)-RH-150721	100.3	90.6
(S)-RH-150721	105.7	93.9
(R)-RH-141288	88.0	95.8
(S)-RH-141288	98.8	92.8
RH-129151 (A)	95.5	81.5
RH-129151 (B)	92.9	84.3
RH-24549	74.7	103.6
RH-141452	78.4	109.6
Hydrolysed samples		
RH-141452	70.1	84.1
Grape juice		
Not hydrolysed samples		
(R)-Zoxamide	109.1	99.0
(S)-Zoxamide	107.8	103.8
(R)-RH-150721	104.4	107.5
(S)-RH-150721	108.9	108.4
(R)-RH-141288	93.0	82.4
(S)-RH-141288	118.8	113.6
RH-129151 (A)	101.3	83.1
RH-129151 (B)	100.6	88.3
RH-24549	99.4	101.1
RH-141452	101.9	101.7
Hydrolysed samples		
RH-141452	80.1	95.0
Wine		
Not hydrolysed samples		
(R)-Zoxamide	108.0	100.9
(S)-Zoxamide	101.7	99.2
(R)-RH-150721	121.7	118.5
(S)-RH-150721	114.8	124.4
(R)-RH-141288	105.3	108.0
(S)-RH-141288	85.6	104.0
RH-129151 (A)	100	70.7

RH-129151 (B)	99.3	71.8
RH-24549	103.7	107.5
RH-141452	106.7	109.9
Hydrolysed samples		
RH-141452	85.5	97.3

All recovery values were between 70-110%.

Table A 5: Recovery results in grapes (BPL-STUDY-000058)

Analyte	Recovery (%)					
	LOQ	10x LOQ	LOQ	10x LOQ	LOQ	10xLOQ
	Table grape				Raisin	
Not hydrolysed samples						
Zoxamide (R)	107.9	101.1	107.8	104.6	96.6	91.5
Zoxamide (S)	109.8	106.7	107.4	108.0	98.0	97.1
RH-141288 (R)	102.0	105.0	95.3	96.0	105.5	98.0
RH-141288 (S)	97.2	95.7	97.0	103.1	85.9	95.3
RH-150721 (R)	147.3	133.1	109.9	109.1	99.6	102.3
RH-150721 (S)	162.5	140.8	106.5	106.0	100.6	93.2
RH-129151 (A)	102.6	109.5	105.8	98.5	93.3	87.0
RH-129151 (B)	91.3	91.7	100.9	84.1	106.8	88.9
RH-24549	105.08	104.98	88.9	98.9	77.8	91.7
RH-141452	104.39	102.63	83.9	95.5	83.5	91.7
Hydrolysed samples						
RH-141452	90.5	89.26	86.4	88.7	84.3	83.4

All recovery values were between 70-110%.

Table A 6: Recovery results in grapes and raisins (18097-03R)

Analyte	Matrix	Fortification level (mg/kg or mg/L)	Recoveries			Overall mean recovery ± RSD %
			Single values (%)	Mean (%)	RSD (%)	
qualifier transition						
(R)-Zoxamide	Grape bunches	0.0050	109.2, 111.3, 112.1, 110.0, 94.7, 88.8	104.3	9.6	99.6 ± 8.4
		0.050	102.9, 101.8, 108.2, 98.9, 97.2	101.8	4.2	
		1.5	94.5, 91.4, 93.4, 90.6, 88.7	91.8	2.5	
	Raisin	0.0050	89.1, 97.0, 89.7, 97.6, 86.5	92.0	5.4	82.7 ± 12.3
		0.050	86.0, 84.4, 86.0, 86.7, 87.6	86.2	1.4	
		0.60	68.9, 75.0, 66.5, 68.7, 70.7	70.0	4.6	
(S)-Zoxamide						
(S)-Zoxamide	Grape bunches	0.0050	112.9, 116.2, 112.3, 105.9, 103.6, 89.4	106.7	9.1	99.9 ± 8.9
		0.050	103.1, 96.5, 104.8, 97.7, 98.7	100.2	3.6	

		1.5	91.6, 93.9, 93.6, 89.4, 88.9	91.5	2.5	
	Raisin	0.0050	87.4, 94.1, 87.1, 97.9, 93.3	92.0	5.1	82.8 ± 12.3
		0.050	86.8, 87.1, 86.7, 86.8, 85.5	86.6	0.7	
		0.60	68.4, 74.8, 66.3, 68.8, 71.2	69.9	4.7	
(R)- RH-150721						
(R)-RH-150721	Grape bunches	0.0050	106.3, 83.7, 84.8, 79.9, 84.1, 95.9	89.1	11.2	92.8 ± 9.7
		0.050	98.3, 89.3, 105.7, 96.9, 95.5	97.1	6.1	
	Raisin	0.0050	63.7, 69.4, 69.0, 72.0, 75.6	69.9	6.3	74.9 ± 8.4
		0.050	76.7, 81.7, 77.0, 82.4, 81.4	79.8	3.4	
(S)-RH-150721						
(S)-RH-150721	Grape bunches	0.0050	102.8, 71.2, 79.4, 84.6, 74.6, 99.0	85.3	15.2	90.3 ± 13.1
		0.050	98.0, 86.8, 107.4, 95.0, 94.0	96.2	7.8	
	Raisin	0.0050	68.0, 72.4, 70.0, 70.0, 77.4	71.6	5.1	73 0 ± 4.2
		0.050	72.3, 74.8, 73.2, 75.5, 76.5	74.5	2.3	
(R)-RH-141288						
(R)-RH-141288	Grape bunches	0.0050	104.3, 111.0, 116.7, 95.0, 100.1, 84.6	101.9	11.2	100.1 ± 8.9
		0.050	105.5, 96.2, 92.1, 96.5, 98.9	97.8	5.0	
	Raisin	0.0050	94.4, 97.3, 94.8, 91.2, 91.5	93.8	2.7	91.3 ± 3.6
		0.050	89.2, 89.6, 89.7, 88.4, 86.5	88.7	1.5	
(S)-RH-141288						
(S)-RH-141288	Grape bunches	0.0050	116.0, 120.2, 112.2, 101.5, 104.2, 87.2	106.9	11.2	105.2 ± 9.9
		0.050	100.2, 96.3, 116.9, 107.6, 94.7	103.2	8.9	
	Raisin	0.0050	97.7, 98.7, 95.2, 97.8, 95.4	97.0	1.6	91.4 ± 6.5
		0.050	84.9, 86.5, 87.2, 86.0, 84.9	85.9	1.1	
(A)-RH-129151						
(A)-RH-129151	Grape bunches	0.0050	120.2, 104.8, 110.4, 81.4, 87.7	100.9	15.9	100.0 ± 15.5
		0.050	93.2, 122.9, 76.6, 102.5, 100.4	99.1	16.9	
	Raisin	0.0050	77.0, 77.0, 82.8, 78.9, 88.3	80.8	6.0	76.2 ± 9.4
		0.050	67.6, 74.7, 62.5, 75.5, 77.4	71.5	8.8	
(B)-RH-129151						
(B)-RH-129151	Grape bunches	0.0050	112.3, 109.3, 103.2, 77.7, 86.0	97.7	15.5	98.6 ± 15.3
		0.050	91.2, 121.1, 76.8, 108.3, 99.8	99.4	16.9	
	Raisin	0.0050	84.4, 83.3, 83.3, 80.5, 88.4	84.0	3.4	77.6 ± 10.8
		0.050	67.2, 74.3, 61.0, 75.5, 77.7	71		
RH-24549						
RH-24549	Grape bunches	0.010	111.1, 113.1, 97.5, 104.5, 108.6	106.9	5.8	107.5 ± 6.3
		0.10	115.1, 110.0, 115.5, 102.1, 97.6	108.1	7.4	
	Raisin	0.010	91.1, 89.3, 92.8, 89.4, 99.8	92.5	4.7	98.1 ± 7.0
		0.10	104.0, 107.5, 103.7, 104.5, 99.2	103.8	2.9	

Free RH-141452						
Free RH-141452	Grape bunches	0.010	104.5, 115.3, 115.3, 113.6, 108.9	111.6	4.3	110.9 ± 5.9
		0.10	116.8, 113.6, 118.7, 101.5, 100.8	110.3	7.7	
	Raisin	0.010	105.3, 104.3, 114.0, 110.7, 115.9	110.0	4.7	109.1 ± 3.8
		0.10	106.0, 113.2, 108.7, 108.6, 104.5	108.2	3.1	
Total RH-141452						
Total RH-141452	Grape bunches	0.010	87.2, 97.9, 91.2, 90.9, 87.9	91.0	4.6	101.5 ± 11.4
		0.10	111.6, 107.7, 115.0, 112.8, 112.6	112.0	2.4	
	Limpid juice	0.010	104.0, 94.6, 104.4, 105.2, 89.3	99.5	7.2	100.6 ± 5.2
		0.10	105.4, 98.8, 101.2, 102.5, 100.3	101.6	2.5	

For the following analytes and matrices, the mean recovery was > 110 %:
- Free RH-141452 in grape at 0.01 mg/kg
- Total RH-141452 in grape at 0.1 mg/kg

Table A 7: Recovery results in grapes and processed commodities (19200-01R)

Analyte	Matrix	Fortification level (mg/kg or mg/L)	Recoveries			Overall mean recovery ± RSD %
			Single values (%)	Mean (%)	RSD (%)	
qualifier transition						
(R)-Zoxamide	Grapevine bunches	0.0050	109.2, 111.3, 112.1, 110.0, 94.7, 88.8	104.3	9.6	99.6 ± 8.4
		0.050	102.9, 101.8, 108.2, 98.9, 97.2	101.8	4.2	
		1.5	94.5, 91.4, 93.4,90.6, 88.7	91.8	2.5	
	Limpid juice	0.0050	106.1, 104.0, 103.7, 106.1, 103 6	104.7	1.2	101.5 ± 3.7
		0.050	100.4, 97.7, 98.3, 99.6, 95.4	98.3	2.0	
	Must	0.0050	97.9, 104.7, 103.7, 105.1, 104.2	103.1	2.9	98.5 ± 6.7
		0.050	95.4, 90.1, 85.5, 93.4, 88.5	90.6	4.3	
		0.6	103.1, 104.4, 96.4, 102.1, 102.6	101.7	3.1	
	Young wine	0.0050	101.6, 98.1, 104.5, 103.1, 113.6	104.2	5.5	98.1 ± 5.9
		0.050	97.3, 92.2, 93.9, 90.3, 97.8	94.3	3.4	
		0.20	94.1, 97.9, 95.5, 93.8, 98.6	96.0	2.3	
	Bottled wine	0.0050	109.7, 116.0, 108.7, 106.9, 118.2	111.9	4.4	103.6 ± 7.1
		0.050	94.5, 103.7, 97.2, 96.1, 105.0	99.3	4.7	
		0.20	95.6, 96.9, 101.1, 100 2, 103.7	99.5	3.3	
(S)-Zoxamide						
(S)-Zoxamide	Grapevine bunches	0.0050	112.9, 116.2, 112.3, 105.9, 103.6, 89.4	106.7	9.1	99.9 ± 8.9
		0.050	103.1, 96.5, 104.8, 97.7, 98.7,	100.2	3.6	
		1.5	91.6, 93.9, 93.6, 89 4, 88.9	91.5	2.5	
	Limpid juice	0.0050	107.8, 101.3, 100.3, 105.7, 105.6	104.1	3.1	100.9 ± 4.2
		0.050	99.7, 98.1, 96.6, 99.3, 94.6	97.7	2.1	
	Must	0.0050	99.4, 107.8, 103.7, 103.5, 104.6	103.8	2.9	99.4 ± 6.7

		0.050	95.8, 89.6, 86.5, 96.2, 89.0	91.4	4.7	
		0.6	105.4, 104.5, 97.6, 1.003.5, 103.7	102.9	3.0	
	Young wine	0.0050	105.5, 99.5, 99.3, 103.2, 111.0	103.7	4.7	97.3 ± 5.9
		0.050	95.7, 90.5, 95.73.6, 89.2, 96.3	93.5		
		0.20	91.9, 95.3, 95.4, 93.5, 97.2	94.7	2.1	
	Bottled wine	0.0050	115.5, 113.2, 107.1, 107.0, 114.5	111.5	3.7	103.9 ± 6 3
		0.050	96.7, 103.5, 100.8, 96.1, 105.9	100.6	4.2	
		0.20	96.4, 97.7, 100.9, 100.6, 102.8	99.7	2.6	
(R)- RH-150721						
(R)-RH-150721	Grapevine bunches	0.0050	106.3, 83.7, 84.8, 79.9, 84.1, 95.6	89.1	11.2	92.8 ± 9.7
		0.050	98.3, 89.3, 105.7, 96.9, 95.5	97.1	6.1	
	Limpid juice	0.0050	101.9, 100.2, 95.6, 97.6, 100.8	99.2	2.6	95.6 ± 4.6
		0.050	96.9, 94.4, 88.6, 93.3, 90.1	92.7	3.6	
	Must	0.0050	101.5, 98.7, 97.2, 90.8, 96 7	97.0	4.1	93.0 ± 7.7
		0.050	100.8, 89.2, 83.7, 90.8, 80.8	89.0	8.6	
	Young wine	0.0050	79.0, 92.3, 94.3, 94.3, 101.7	92.3	8.9	89.8 ± 7.2
		0.050	89.7, 86.1, 85.3, 83.6, 91.3	87.2	3.7	
	Bottled Wine	0.0050	107.8, 112.5, 103.8, 102.5	104.6	5.8	98.8 ± 7.7
		0.050	90.6, 96.8, 95.5, 92.3, 90.1	93.1	3.2	
(S)-RH-150721						
(S)-RH-150721	Grape bunches	0.0050	102.8, 71.2, 79.4, 84.6, 74.6, 99.0	85.3	15.2	90.3 ± 13.1
		0.050	98.0, 86.8, 107.4, 95.0, 94.0	96.2	7.8	
	Limpid juice	0.0050	102.2, 99.5, 103.2, 102.1, 101.9	101.8	1.4	96.8 ± 5.8
		0.050	94.5, 92.3, 90.4, 93.4, 88.2	91.8	2.7	
	Must	0.0050	101.3, 104.3, 102.4, 99.4, 88.1	99.1	6.5	93.9 ± 9.7
		0.050	103.4, 87.9, 82.9, 88.6, 80.9	88.7	10.0	
	Young wine	0.0050	100.8, 88.1, 98.1, 99.6, 100.4	97.4	5.5	91.0 ± 9.4
		0.050	94.6, 78.3, 83.2, 83.5, 83.8	84.7	7.0	
	Bottled wine	0.0050	104.3, 109.6, 100.8, 99.4, 92.7	101.4	6.2	97.5 ± 6.2
		0.050	93.7, 92.9, 95.8, 95.1, 90.4	93.6	2.2	
(R)-RH-141288						
(R)-RH-141288	Grape bunches	0.0050	104.3, 111.0, 116.7, 95.0, 100.1, 84.6	101.9	11.2	100.1 ± 8.9
		0.050	105.5, 96.2, 92.1, 96.5, 98.9	97.8	5.0	
	Limpid juice	0.0050	105.1, 104.7, 103.7, 108.5, 98.5	104.1	3.5	100.7 ± 4.6
		0.050	100.2, 98.4, 94.7, 98.4, 94.4	97.2	2.6	
	Must	0.0050	102.7, 104.1, 104.3, 102.3, 98.2	102.3	2.4	96.3 ± 7.4
		0.050	96.7, 88.5, 87.6, 91.8, 86.6	90.3	4.6	
	Young wine	0.0050	88.2, 96.5, 94.2, 97.3, 93.8	94.0	3.8	90.7 ± 4.8
		0.050	86.8, 89.4, 87.5, 86.8, 86.2	87.4	1.4	

	Bottled wine	0.0050	109.2, 112.1, 106.1, 106.6, 118.1	110.4	4.4	104.4 ± 8.0
		0.050	93.9, 100.3, 94.8, 93.8, 108.8	98.3	6.6	
(S)-RH-141288						
(S)-RH-141288	Grape bunches	0.0050	116.0, 120.2, 112.2, 101.5, 104.2, 87.2	106.9	11.2	105.2 ± 9.9
		0.050	100.2, 96.3, 116.9, 107.6, 94.7	103.2	8.9	
	Limpid juice	0.0050	105.3, 99.0, 101.4, 99.5, 104.4	101.9	2.8	100.2 ± 2.9
		0.050	100.5, 98.1, 97.8, 99.8, 95.8	98.1	1.9	
	Must	0.0050	99.3, 104.4, 105.1, 97.7, 103.9	102.1	3.3	95.3 ± 8.5
		0.050	94.8, 88.1, 85.3, 90.8, 83.5	88.5	5.1	
	Young wine	0.0050	98.0, 105.9, 104.5, 100.4, 104.1	102.6	3.2	98.6 ± 5.0
		0.050	93.3, 93.0, 96.2, 92.9, 98.2	94.7	2.5	
	Bottled wine	0.0050	105.2, 120.6, 109.2, 105.2, 118.0	111.6	6.5	105.4 ± 8.3
		0.050	92.9, 103.9, 99.0, 95.8, 104.2	99.2	5.0	
(A)-RH-129151						
(A)-RH-129151	Grape bunches	0.0050	120.0, 104.8, 110.4, 81.4, 87.7	100.9	15.9	100.0 ± 15.5
		0.050	93.2, 122.9, 76.6, 102.5, 100.4	99.1	16.9	
	Limpid juice	0.0050	119.7, 117.1, 111.1, 103.8, 125.6	115.5	7.2	109.2 ± 10.2
		0.050	113.7, 103.4, 86.1, 101.2, 110.7	103.0	10.5	
	Must	0.0050	106.6, 106.0, 105.3, 103.7, 99.9	104.3	2.6	101.3 ± 5.3
		0.050	104.5, 101.9, 89.2, 99.8, 96.3	98.3	6.0	
	Young wine	0.0050	72.5, 77.3, 64.2, 79.4, 100.8	78.8	17.3	70.9 ± 18.3
		0.050	60.5, 70.4, 63.8, 54.7, 65.2	62.9	9.2	
	Bottled wine	0.0050	123.1, 113.3, 116.2, 115.6, 133.3	120.3	6.8	113.7 ± 8.9
		0.050	103.7, 102.5, 119.7, 102.0, 107.9	107.1	6.9	
(B)-RH-129151						
(B)-RH-129151	Grape bunches	0.0050	112.3, 109.3, 103.2, 77.7, 86.0	97.7	15.5	98.6 ± 15.3
		0.050	91.2, 121.1, 76.8, 108.3, 99.8	99.4	16.9	
	Limpid juice	0.0050	118.6, 116.9, 114.5, 105.4, 128.9	116.9	7.2	110.3 ± 10.2
		0.050	114.0, 100.7, 87.6, 106.7, 109.2	103.6	9.8	
	Must	0.0050	100.4, 104.2, 102.8, 98.8, 102.3	101.7	2.1	100.6 ± 4.3
		0.050	105.1, 102.1, 89.9, 102.4, 98.5	99.6	5.9	
	Young wine	0.0050	72.4, 80.5, 63.5, 89.4, 101.8	81.5	18.2	72.0 ± 20.1
		0.050	58.4, 67.2, 66.7, 56.3, 63.9	62.5	7.9	
	Bottled Wine	0.0050	110.7, 105.2, 126.0, 124.4, 132.8	119.8	9.5	114.0 ± 9.8
		0.050	102.2, 101.6, 119.7, 104.1, 113.8	108.3	7.4	
RH-24549						
RH-24549	Grape bunches	0.010	111.1, 113.1, 97.5, 104.5, 108.6	106.9	5.8	107.5 ± 6.3
		0.10	115.1, 110.0, 115.5, 102.1, 97.6	108.1	7.4	
	Limpid juice	0.010	115.8, 105.3, 99.6, 116.6, 112.8	110.0	6.7	112.3 ± 5.7

		0.10	108.0, 111.9, 117.4, 120.4, 115.4	114.6	4.2	
	Must	0.010	78.2, 84.1, 115.3, 115.1, 118.3	102.2	19.0	106.6 ± 13.1
		0.10	114.7, 105.9, 109.8, 113.8, 110.7	111.0	3.2	
	Young wine	0.010	101.0, 105.5, 118.1, 113.8, 121.3	112.0	7.6	110.9 ± 6.1
		0.10	103.2, 108.9, 107.4, 112.4, 117.3	109.8	4.8	
	Bottled wine	0.010	108.2, 110.8, 114.8, 124.3, 119.0	115.4	5.6	113.2 ± 5.6
		0.10	103.1, 108.7, 109.3, 115.8, 118.2	111.0	5.4	
Free RH-141452						
Free RH-141452	Grape bunches	0.010	104.5, 115.3, 115.5, 113.6, 108.9	111.6	4.3	110.9 ± 5.9
		0.10	116.8, 113.6, 118.7, 101.5, 100.8	110.3	7.7	
	Limpid juice	0.010	107.7, 108.3, 103.8, 108.7, 116.5	109.0	4.2	112.2 ± 4.4
		0.10	115.9, 117.5, 113.0, 118.7, 112.2	115.5	2.4	
	Must	0.010	107.0, 102.2, 103.0, 104.7, 108.6	105.1	2.6	106.0 ± 2.6
		0.10	110.0, 108.1, 106.5, 107.9, 102.5	107.0	2.6	
	Young wine	0.010	87.2, 102.8, 109.2, 104.3, 116.9	104.1	10.5	105.7 ± 7.4
		0.10	104.5, 112.9, 106.2, 109.1, 104.0	107.3	3.4	
	Bottled wine	0.010	104.6, 107.2, 103.2, 107.8, 104.8	105.5	1.8	108.4 ± 3.6
		0.10	111.5, 110.9, 111.7, 115.5,106.8	111.3	2.8	
Total RH-141452						
Total RH-141452	Grape bunches	0.010	87.2, 97.9, 91.2, 90.9, 87.9	91.0	4.6	101.5 ± 11.4
		0.10	111.6, 107.7, 115.0, 112.8, 112.6	112.0	2.4	
	Limpid juice	0.010	110.7, 112.5, 118.8, 111.8, 118.6	114.5	3.4	110.0 ± 4.4
		0.10	102.8, 108.2, 107.6, 107.7, 110.8	107.4	2.7	
	Must	0.010	114.2, 117.9, 119.3, 111.3, 118.5	116.2	2.9	112.8 ± 4.0
		0.10	109.6, 107.1, 111.3, 107.1, 111.7	109.4	2.0	
	Young wine	0.010	131.5, 119.3, 104.0, 97.0, 115.8	113.5	11.8	110.6 ± 9.3
		0.10	110.4, 111.9, 100.5, 102.0, 113.4	107.7	5.5	
	Bottled wine	0.010	110.9, 101.5, 109.0, 109.0, 108.3	107.7	3.4	106.5 ± 2.8
		0.10	103.6, 107.1, 106.3, 103.6, 105.8	105.3	1.5	

For the following analytes and matrices, the mean recovery was > 110 %:

- (R)-zoxamide, (S)-zoxamide and (S)-RH-141288 at 0.005 mg/L in bottled wine
- (A) RH-129151 and (B) RH-129151 at 0.005 mg/L in limpid juice and bottled wine
- RH-24549 at 0.01 mg/L in young wine and bottled wine; at 0.1 mg/kg-L in limpid juice, must and bottled wine
- Free RH-141452 at 0.01 mg/kg in grape; at 0.1 mg/L in limpid juice and bottled wine
- Total RH-141452: at 0.01 mg/Kg-L in limpid juice, in must and in young wine; at 0.1 mg/kg in grape

For the following analytes and matrices, the mean recovery was < 70 % (see deviation, chapter 15):

- (A) RH-129151 and (B) RH-129151 at 0.05 mg/L in young wine.

Storage stability of sample extracts

Study no.	Max. storage of sample extracts
AB2-18-35355	within 24 hours*
BPL-STUDY-19-000041	Zoxamide and metabolites within 3 days RH-129151 within 24 hours
BPL-STUDY-19-000051	Zoxamide and metabolites within 3 days RH-129151 within 24 hours
BPL-STUDY-19-000057	Zoxamide and metabolites within 3 days RH-129151 within 24 hours
BPL-STUDY-19-000058	Zoxamide and metabolites with-in 3 days
18097-03R	Within 24 hours *
17120-01R	Within 24 hours *
17120-02R	Within 24 hours *
19200-01R	Within 24 hours *

* No storage stability performed.

The stability of the analytes in the final extracts kept at 4°C for 3 days was successfully verified for all the analytes except RH-129151 in the GLP study no. BPL-STUDY-18-000085. For RH-129151 the stability of the residue in the sample extract over a period of 2 days has been successfully demonstrated by Longhi (2021) in study GLP-STUDY-20-77.

Freezer storage of samples

The maximal storage interval between sampling and analysis was:

Study no.	Residue	Commodity	Max. storage of sample extracts
AB2-18-35355	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Bunches, juice, must, young wine, bottled wine	458 - 495 days
BPL-STUDY-19-000041	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Berries, juice, young wine, stored wine	Barriers: 640 – 643 days Juice: 626-632 days Young wine: 585-591 days Stored wine: 400-406 days
BPL-STUDY-19-000051	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Berries, juice, young wine, bottled wine	Barriers: 630 – 634 days Juice: 627-629 days Young wine: 480-482 days Bottled wine: 343-345 days
BPL-STUDY-19-000057	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Grape wine	42 – 45 day*s
BPL-STUDY-19-000058	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Table grape, raisin	6 – 26 days
18097-03R	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Table grape, raisin	Bunches: 481 days Raisin: 484 days
17120-01R	Zoxamide	Table grape	Max. 133 days
17120-02R	Zoxamide	Table grape, juice, must, young and bot-tled wine	Max. 287 day

19200-01R	Zoxamide, RH-141452, RH-24549, RH-150721, RH-129151, RH-141288	Berries, juice, young wine, bottled wine	Berries: 821 - 846 days Juice: 846 days Must: 853 days Young wine: 743 days Bottled wine: 660 days

* Note: a maximum time of 83 days elapsed for all the analytes for RETAIN samples analysed to confirm SHIP samples results (this has no influence on the maximum storage time values since only the results of SHIP samples are reported in the TIER summaries)

Conclusion

The recovery results comply with the acceptance criterium of SANCO 825/00, which demands a mean recovery for each matrix and at each fortification level in the range of 70–120%. The relative standard deviations for each fortification level were ≤ 20 %. Therefore, the method BPL-STUDY-18-000085 permits the determination of residues of zoxamide and its metabolites in grapes and its processed fractions with satisfactory accuracy and precision. In addition, the method is regarded as highly specific, using Multi Reaction Monitoring (MRM) with two mass transitions.

The limit of quantification (LOQ) was set at 0.01 mg/kg for zoxamide, RH-150721, RH-129151 and RH-141288 (sum of enantiomers), RH-24549, free RH-141452 and total RH-141452, and 0.005 mg/kg for single isomers. The limit of determination (LOD) was given as 0.003 mg/kg for RH-150721, RH-129151 and RH-141288 (sum of enantiomers) and RH-24549, free RH-141452 and total RH-141452 and 0.0015 mg/kg for single isomers.

(Peterek S. 2020 ff.)

A 2.1.1.1.2 Analytical method 2 – determination of zoxamide and benalaxyl-m on grapes (AGRI BPL 064 rev. 1)

A 2.1.1.1.2.1 Method validation

Comments of zRMS:	The method is accepted. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/12**
Report	Luciani, G.P., 2016: Determination of zoxamide and benalaxyl-m residues after three applications of GWN-10392 on wine grapes under field conditions – Italian trial, year 2015 Gowan Comércio Internacional e Servicos, Limitada, Portugal Tentamus AgriParadigma srl, Italy, Report No. AGRI 009/15 GLP HAR, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

An LC-MS/MS method for the determination of zoxamide and benalaxyl-m residues in wine grapes, limpid juice, must and wine (method Agri BPL 064 Rev.1) has been validated according to SANCO/3029/99 rev. 4 under report no. Agri BPL 064 rev. 1.

Weigh 10.0 + 0.1 g for sample, previously homogenised, into 50 ml Teflon centrifuge tube. Add 10 ml acetonitrile, shake sample vigorously for 1 min using Vortex mixer at maximum speed. Add 4 g anhydrous $MgSO_4$, 1g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate and mix on a Vortex mixer immediately for 5 min and centrifuge for 5 min at > 3000 g. The final extract was directly employed for LC determinative analysis. Benalaxyl-m and zoxamide residues were quantified by high performance liquid chromatography with external standard calibration. The analytes were detected with a triple quadrupole mass spectrometer operating in Multiple Reaction Monitoring (MRM).

Limit of analytical quantification (LOQ) of benalaxyl-m and zoxamide in wine grapes, limpid juice, must and wine was 0.01 mg/kg.

Equipment

LC-MS/MS System: Waters ACQUITY UPLC System with 4-PREMIER
Column: Waters Acquity UPLC HSS T3. 1.8µm 2.1 x 100mm
Mobile phase: A: Methanol 10% + Water 90% + 0.005M Ammonium Formiate ($HCOONH_4$)
B: Methanol + 0.005 M ammonium Formiate ($HCOONH_4$)

Total time (min)	A (%)	B (%)
0.25	99.9	0.1
7.75	0.1	99.9
9	0.1	99.9
9.01	99.9	0.1
10.0	99.9	0.1

Flow rate: 450µL/min
Column temp.: 40°C
Injection volume: 10 µL
Retention time: ~ 7.0 min for zoxamide and Benalaxyl-M
Ionisation: MRM (Positive Multiple reaction Monitoring)
Ion mode
Zoxamide:
m/z 336 → m/z 187 (quantitative MRM)
m/z 336 → m/z 159 (confirmative MRM)
Benalaxyl-M:
m/z 236.1 → m/z 148 (quantitative MRM)
m/z 236.1 → m/z 91 (confirmative MRM)

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 8: Recoveries after fortification with zoxamide

Fortification level (F)		Response R	Concentration C	Residue found RF=C*V/W*d	Mean value
	(mg/kg)	(Area)	(mg/L)	(mg/kg)	(%)
Wine grape	0.0108	62336	0.0090	0.0090	82.3
	0.0108	60393	0.0087	0.0087	

	0.0108	61794	0.0089	0.0089	
	0.0108	61894	0.0090	0.0090	
	0.0108	60598	0.0087	0.0087	
	0.154	675707	0.137	0.137	
	0.154	665780	0.135	0.135	
	0.154	675986	0.137	0.137	89.1
	0.154	683361	0.139	0.139	
	0.154	677152	0.137	0.137	
Limpid juice	0.0108	64567	0.0095	0.0095	
	0.0108	63468	0.0093	0.0093	
	0.0108	65622	0.0097	0.0097	88.0
	0.0108	64339	0.0094	0.0094	
	0.0108	64988	0.0095	0.0095	
	0.154	795319	0.163	0.163	
	0.154	796154	0.163	0.163	
	0.154	791435	0.162	0.162	105.4
	0.154	799239	0.163	0.163	
	0.154	784097	0.160	0.160	
Must	0.0092	55000	0.0084	0.0084	
	0.0092	56707	0.0086	0.0086	
	0.0092	56867	0.0087	0.0087	93.4
	0.0092	57233	0.0087	0.0087	
	0.0092	56899	0.0087	0.0087	
	0.154	665669	0.134	0.134	
	0.154	658318	0.133	0.133	
	0.154	666213	0.134	0.134	86.7
	0.154	658327	0.133	0.133	
	0.154	661635	0.133	0.133	
Wine	0.0092	57225	0.0087	0.0087	
	0.0092	57607	0.0088	0.0088	
	0.0092	57426	0.0088	0.0088	94.5
	0.0092	57389	0.0088	0.0088	
	0.0092	55997	0.0085	0.0085	
	0.154	662778	0.133	0.133	
	0.154	665903	0.134	0.134	
	0.154	677404	0.137	0.137	88.2
	0.154	676304	0.136	0.136	
	0.154	681905	0.138	0.138	

Initial sample weight (W): 10g
Sample final volume (V): 10 Acetonitrile

Table A 9: Recoveries after fortification with benalaxyl-m

Fortification level (F)		Response R	Concentration C	Residue found RF=C*V/W*d	Mean value
	(mg/kg)	(Area)	(mg/L)	(mg/kg)	(%)
Wine grape	0.0109	130218	0.0092	0.0092	
	0.0109	131418	0.0093	0.0093	87.4
	0.0109	134265	0.0096	0.0096	

	0.0109	137037	0.0098	0.0098	
	0.0109	137617	0.0099	0.0099	
	0.156	1593434	0.152	0.152	
	0.156	1578731	0.151	0.151	
	0.156	1616707	0.154	0.154	98.2
	0.156	1632880	0.156	0.156	
	0.156	1608626	0.153	0.153	
	0.0109	135586	0.0097	0.0097	
	0.0109	139268	0.0101	0.0101	
	0.0109	136316	0.0098	0.0098	90.4
	0.0109	138217	0.0100	0.0100	
Limpid juice	0.0109	137672	0.0099	0.0099	
	0.156	1766266	0.169	0.169	
	0.156	1808494	0.173	0.173	
	0.156	1772892	0.170	0.170	109.6
	0.156	1797337	0.172	0.172	
	0.156	1798557	0.172	0.172	
	0.0094	91828	0.0073	0.0073	
	0.0094	104125	0.0083	0.0083	
	0.0094	109447	0.0087	0.0087	89.9
	0.0094	111050	0.0089	0.0089	
Must	0.0094	111028	0.0089	0.0089	
	0.156	1454411	0.135	0.135	
	0.156	1419466	0.132	0.132	
	0.156	1395601	0.129	0.129	85.7
	0.156	1463377	0.136	0.136	
	0.156	1477485	0.137	0.137	
	0.0094	108290	0.0087	0.0087	
	0.0094	110703	0.0088	0.0088	
	0.0094	108467	0.0087	0.0087	92.9
	0.0094	104978	0.0084	0.0084	
Wine	0.0094	107737	0.0086	0.0086	
	0.156	1356009	0.125	0.125	
	0.156	1350535	0.125	0.125	
	0.156	1348156	0.125	0.125	80.5
	0.156	1342583	0.124	0.124	
	0.156	1390541	0.129	0.129	

Initial sample weight (W): 10g
Sample final volume (V): 10 Acetonitrile

Accuracy and precision / repeatability

The accuracy of the method was assessed on the basis of the determined recovery rates. The accuracy of the method fulfils the requirements for residue analytical methods which demand that the mean recoveries per fortification level should be in the range 70-110%.

Linearity

Linearity was demonstrated over an calibration range of 0.005 mg/L to 0.25 mg/L for zoxamide and benal-axyl-m.

Limit of quantification (LOQ)

Limit of analytical quantification (LOQ) of benalaxyl-m and zoxamide in wine grapes, limpid juice, must and wine was set at 0.01 mg/kg.

Specificity

The analytes showed typical Rf values. The method can be regarded as specific, monitoring two mass transitions.

Stability of sample extracts and standard solutions

Final sample extracts were analysed within 24 hours.

Freezer storage of samples

The maximal storage interval between sampling and analysis was 167 days.

No MS spectra were provided in the study report.

Table A 10: Characteristics for the analytical method used for the analysis of zoxamide and benalaxyl-m in wine grapes and processed fractions

	Zoxamide	**Benalaxyl-m**
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. 6 point calibration; $r^2 > 0.9967$ y = 4.680.900,7187x + 34.184,0611 Individual calibration data and calibration line equation presented in the study report.	Matrix matched standard calibration. 6 point calibration; $r^2 > 0.9991$ y= 10.244A50,0616x + 36.240,3671 Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.005 - 0.25 mg/L (corresponds to 0.005 – 0.25 mg/kg)	0.005 - 0.25 mg/L (corresponds to 0.005 – 0.25 mg/kg)
Assessment of matrix effects is presented	No (Matrix matched standard calibration)	No (Matrix matched standard calibration)
Limit of quantification (LOQ)	LOQ: 0.010 mg/kg	LOQ: 0.010 mg/kg

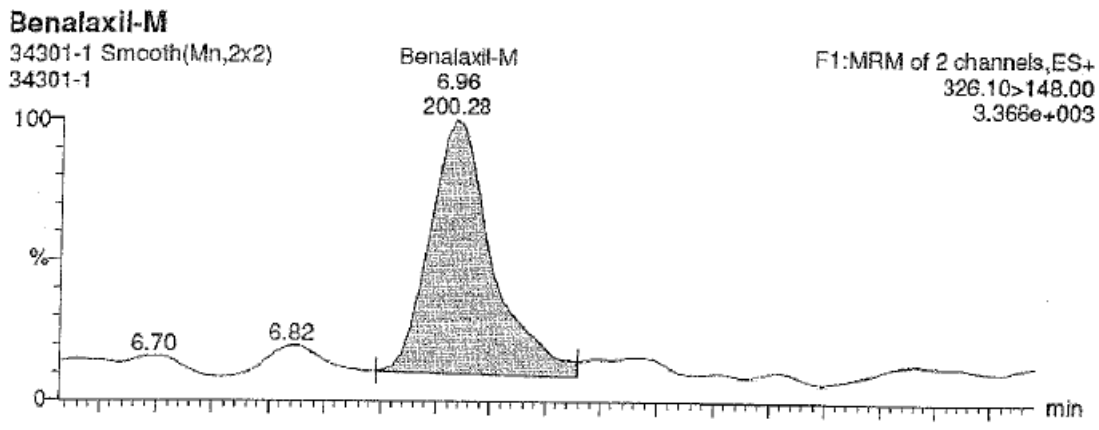


Figure A 1: **Typical chromatogram for benalaxyl-m**

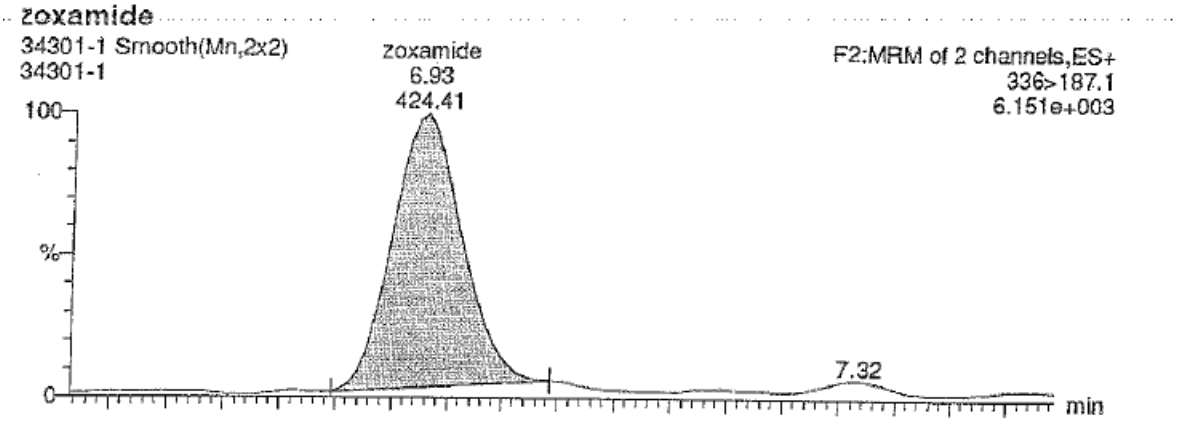


Figure A 2: **Typical chromatogram for zoxamide**

Conclusion

An LC-MS/MS method for the determination of zoxamide and benalaxyl-m residues in wine grapes, limpid juice, must and wine (method Agri BPL 064 Rev.1) with an LOQ of 0.01 mg/kg has been successfully validated according to SANCO/3029/99 rev. 4.

(Luciani G. P. 2016)

A 2.1.1.1.3 Analytical method 3 – determination of zoxamide and benalaxyl-m in grapes and processed commodities (RAU-049-15)

A 2.1.1.1.3.1 Method validation

Comments of zRMS:	Study accepted. ~~For evaluation see Section 7 of the present RR.~~ 2 trials were conducted in Northern France. The analytical method (LC-MS/MS) was validated in terms of accuracy, precision, linearity, selectivity, LOQ and LOD in compliance with guidelines SANCO/825/00 rev. 8.1 (16/11/2010) and SANCO/3029/99, rev. 4 (11/07/2000) by means of recovery tests and analysis of blank samples on bunches, must and wine. A mean recovery of 70-110% with a Relative Standard Deviation lower than 20% was adopted as acceptability criteria. The validation parameters were in required range

Reference:	**KCP 5.1/13**
Report	Perboni, A., 2017: Determination of benalaxyl-m and zoxamide residues in raw agricultural commodity grapes (wine and table) and processed commodity (must, fermenting must, wine and aged wine) following three applications of GWN-10392 (benalaxyl-m 150 g/L + zoxamide 225 g/L) in open field condition (3 harvest trials, northern and southern Europe, year 2015) Gowan Crop Protection Limited, UK BioTecnologie BT Srl., Italy, Report No. RAU-049-15, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)

Deviations:	Deviation during processing phase of trial F/BZ15/GR03: The must commodity was sampled 7 days later than planned since the fermentation did not start in time. This deviation was regarded to have no impact on the results and the integrity of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method has been validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) by means of recovery experiments on blank samples of bunches, must and wine.

An accurate amount (10 g) of each specimen was weighted in a 50 ml centrifuge tube and 10 ml of acetonitrile were added to the sample. The sample was extracted by shaking thoroughly for 3 minutes. After this first extraction step 4 g of magnesium sulphate anhydrous, 1g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1g of sodium citrate tribasic dehydrate were added to the mixture and the tube was further shaken for 3 minutes. The extract was then centrifuged for 5 minutes at 5000 RPM. The supernatant (2 ml) was transferred in a 10 ml centrifuge tube and centrifuged on Spectrafuge 16M centrifuge for 2 minutes. An aliquot of acetonitrile was diluted with water prior to analyse using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Matrix-matched standard solutions were used for analyte determination.

Equipment

LC/MS-MS:	Thermo finnigan TS Quantum LC-MS/MS equipped with an autosampler
Column:	Kinetex 1.7 µm XB C18 50 mm x 2.1 mm Phenomenex
Mobile phase:	Solvent A: water + 0.1% formic acid Solvent B: acetonitrile + 0.1% formic acid

Time [min]	% A	% B
0	95	5
1	95	5
11	5	95
13	5	95
13.2	95	5
15	95	5

Flow rate:	250 µl/min
Column temp.:	40 °C
Injection volume:	2 µL
Run time:	15 min
Retention time:	Benalaxyl-M: approx. 8.75 min Zoxamide: approx. 9.00 min
Ionisation:	ESI, Positive ions
Ion mode (SRM mode):	Zoxamide: m/z 336 → m/z 187 (quantifier ion) m/z 336 → m/z 159 (qualifier ion) Benalaxyl-M:

m/z 326 → m/z 148 (quantifier ion)
m/z 326 → m/z 208 (qualifier ion)

Results and discussions

Table A 11: Recovery results for benalaxyl-m

Matrix	Fortification level (mg/kg)	Accuracy and precision for level		Overall accuracy and precision	
		Mean Recovery (%) n=5	RSD (%) n=5	Mean Recovery (%) n=10	RSD (%) n=10
Grapes	0.01	97.39	2.23	93.90	4.60
	2.0	90.41	2.88		
Must	0.01	96.01	4.02	89.88	8.41
	0.1	83.74	5.28		
Wine	0.01	87.77	11.71	93.54	10.53
	0.1	99.30	5.50		

Table A 12: Recovery results for zoxamide

Matrix	Fortification level (mg/kg)	Accuracy and precision for level		Overall accuracy and precision	
		Mean Recovery (%) n=5	RSD (%) n=5	Mean Recovery (%) n=10	RSD (%) n=10
Grapes	0.01	94.03	5.27	94.14	5.14
	2.0	94.24	5.63		
Must	0.01	92.52	7.17	86.99	9.32
	0.1	81.46	6.43		
Wine	0.01	88.13	13.22	94.07	11.24
	0.1	100.02	5.23		

Accuracy and precision / repeatability

Five recovery tests were performed at each fortification level, 0.01 mg/kg level (LOQ) and 2.0 mg/kg level for both analytes for grapes samples and from 0.01 mg/kg level (LOQ) and 0.1 mg/kg level for both analytes for processed commodities, by adding a known quantity of analytical standards to the control samples. All the recoveries were within the acceptable ranges of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was lower than 20%. The results obtained are in compliance with acceptability criteria reported in SANCO/825/00, rev 8.1.

Linearity

The response was found to be linear in the range of concentration 2.999 µg/L – 499.9 µg/L for benalaxyl-m and from 3.005 µg/L – 500.9 µg/L for zoxamide (5 concentration levels each). The correlation coefficient R^2 was well higher than 0.99 in all cases.

Limit of quantification (LOQ) and limit of detection (LOD)

The limit of quantification was defined as the lowest fortification level at which acceptable recovery was obtained. This level was found at 0.01 mg/kg can be assumed as LOQ for both analytes and all matrices.

The LOD was defined as the smallest measurable concentration at which the signal is higher than at least three times the background noise. The limit of detection (LOD) was found at 0.0030 mg/kg for both analytes and all matrices.

Matrix effects

Two independent analysis of blank sample per matrix were performed. No significant interferences exceeding 30% of the limit of quantification were determined in each sample at the retention time of benalaxyl-m and zoxamide in LC/MS analysis. However, matrix-matched standard solutions were used for analyte determination.

Specificity

The method can be regarded as specific with typical Rf values, typical mass spectra of the analytes and monitoring two ion transitions.

Stability of sample extracts

Final sample extracts were analysed within 24 hours.

Freezer storage of samples

The maximum storage interval between sampling and analysis was 233 days.

Table A 13: Characteristics for the analytical method used for validation of and zoxamide residues in grapes

	Zoxamide	**Benalaxyl-m**
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration. 5 point calibration, r^2 >0.9995; linear Regression Model $y = 1\ e^{-07}x + 0.0028$ Individual calibration data and calibration line equation presented in the study report.	Standard solution and matrix matched calibration. 5 point calibration, r^2 >0.99; linear Regression Model $y = 1\ e^{-08}x + 0.0016$ Individual calibration data and calibration line equation presented in the study report.
Calibration range	3.005 – 500.9 µg/L	2.999 – 499.9 µg/L
Assessment of matrix effects is presented	Matrix matched standard solutions	Matrix matched standard solutions
Limit of determination/quantification	LOQ: 0.01 mg/kg LOD: 0.0030 mg/kg	LOQ: 0.01 mg/kg LOD: 0.0030 mg/kg

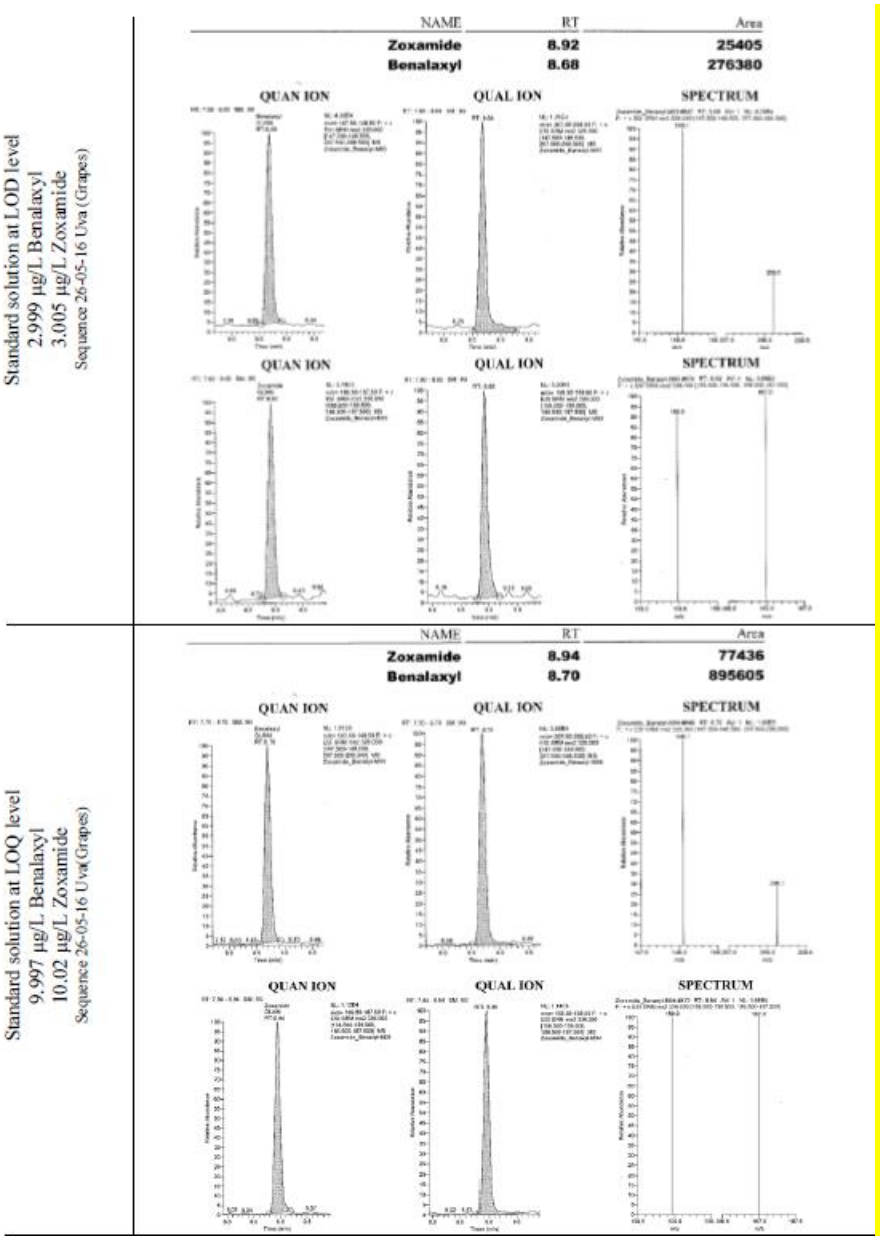


Figure A 3: **Representative chromatograms of zoxamide and benalaxyl-m**

Conclusion

An LC-MS/MS method for the determination of zoxamide and benalaxyl-m residues in wine grapes, limpid juice, must and wine (method Agri BPL 064 Rev.1) has been successfully validated according to SANCO/3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

(Perboni A. 2017)

A 2.1.1.1.4 Analytical method 4 – determination of zoxamide and cymoxanil on grapes and processed fractions

A 2.1.1.1.4.1 Method validation

Comments of zRMS:	The studies 5.1/14, 15 were already provided and assessed during the product authorisation.

Reference:	**KCA 5.1/14**
Report:	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) Following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Northern Europe 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2117, GLP, Not published
Guideline(s):	SANCO/825/00 rev.7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviation(s):	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCA 5.1/15**
Report:	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Northern Europe 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2120, GLP, Not published
Guideline(s):	SANCO/825/00 rev.7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviation(s):	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

A sample of bunches was taken and analyzed straight after deep freezing. For cymoxanil the sample was extracted by Ultra-Turrax (bunch samples) or by shaking (processed samples) with ethyl acetate, purified by liquid-liquid partition and analysed by gas chromatography with a Nitrogen Phosphorus Detector (GC/NPD). Zoxamide was determined using an internal analytical method that consisted of a solvent extraction by Ultraturrax (bunch samples) or by shaking (processed samples). The extract containing the active ingredient was cleaned up by liquid-liquid partition and by SPE chromatography, then analysed by gas chromatography equipped with an ECD. The Limit of Quantification (LOQ) was 0.05 mg/kg for cymoxanil on bunches samples and 0.01 mg/kg on processed samples; for zoxamide the LOQ was 0.01 mg/kg for all matrices. Limit of Detection (LOD) was 0.0290 mg/kg for bunch samples and 0.0058 mg/kg for processed samples (must, young wine and bottled wine) for cymoxanil, and 0.005 mg/kg for zoxamide, all matrices.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.25175 µg/mL to 2.51750 µg/mL (cymoxanyl) and 0.01019 µg/mL to 0.1019 µg/mL (zoxamide). The linear correlation was calculated for each analyte in wine matrix:

- Cymoxanil
  r2 =0.999939 Y= 0.025487X+0.061535 (n= 6)
- Zoxamide
  r2 = 0.99006 Y= 0.000011X+0.006646 (n= 6)

Specificity

The method was regarded as specific.

Recoveries of zoxamide

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR01/01C/1R	Grapes / Bunch	0.01	74.58
I/CZ10/GR01/01C/2R	Grapes / Bunch	0.1	71.93
I/CZ10/GR01/01C/4R	Grapes / Bunch	0.5	70.70
I/CZ10/GR01/01C/5R	Grapes / Bunch	0.5	86.87
I/CZ10/GR01/01C/6R	Grapes / Bunch	0.5	85.22
I/CZ10/GR04/09C/1R	Grapes / Bunch	0.01	72.62
I/CZ10/GR04/09C/2R	Grapes / Bunch	0.1	77.82
Overall mean recovery			**77.11**
Overall standard deviation			**6.53**
Overall relative standard deviation (RSD)			**8.5**

Table 2: Recovery test result on grapes / bunch samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR02/05C/MU/1R	Must	0.01	86.36
I/CZ10/GR02/05C/MU/2R	Must	0.1	71.54
I/CZ10/GR02/05C/YW/1R	Young Wine	0.01	73.60
I/CZ10/GR02/05C/YW/2R	Young Wine	0.1	95.19
I/CZ10/GR02/05C/BW/1R	Bottled Wine	0.01	79.49
I/CZ10/GR02/05C/BW/2R	Bottled Wine	0.1	73.31
Overall mean recovery			**79.92**
Overall standard deviation			**9.26**
Overall relative standard deviation (RSD)			**11.6**

Table 3: Recovery test result on grapes / processed samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR01/01C/1R	Grapes / Bunch	0.01	74.58
I/CZ10/GR04/09C/1R	Grapes / Bunch	0.01	72.62
Overall mean recovery			**73.60**
Overall standard deviation			**1.39**
Overall relative standard deviation (RSD)			**1.9**

Table 4: Recovery test result on grapes / bunch samples at LOQ (0.01 mg/kg) spiking level

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR02/05C/MU/1R	Must	0.01	88.36
I/CZ10/GR02/05C/YW/1R	Young wine	0.01	73.60
I/CZ10/GR02/05C/BW/1R	Bottled wine	0.01	79.49
Overall mean recovery			79.82
Overall standard deviation			6.39
Overall relative standard deviation (RSD)			8.0

Table 5: Recovery test result on grapes / processed samples at LOQ (0.01 mg/kg) spiking level

Recoveries of cymoxanil

Analytical code	matrix	Cymoxanil	
		Level added (mg/kg)	Recovery %
G/CZ10/GR01/01C/1R	Grapes Bunch	0.05	95.73
G/CZ10/GR01/01C/2R	Grapes Bunch	0.5	87.96
G/CZ10/GR01/01C/3R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/4R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/5R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/6R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR03/07C/1R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR03/07C/2R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR04/09C/1R	Grapes Bunch	0.05	95.13
F/CZ10/GR04/09C/2R	Grapes Bunch	0.5	84.31
Overall mean recovery			**90.78**
Overall standard deviation			**5.57**
Overall relative standard deviation (RSD)			**6.1**

(1) *N.A.: not applicable*

Analytical code	matrix	Cymoxanil	
		Level added (mg/kg)	Recovery %
F/CZ10/GR02/05C/MU/1R	Must	0.01	106.26
F/CZ10/GR02/05C/MU/2R	Must	0.1	106.95
Overall mean recovery			**106.61**
Overall standard deviation			**0.49**
Overall relative standard deviation (RSD)			**0.5**
F/CZ10/GR02/05C/YW/1R	Young Wine	0.01	99.30
F/CZ10/GR02/05C/YW/2R	Young Wine	0.1	94.04
Overall mean recovery			**96.67**
Overall standard deviation			**3.72**
Overall relative standard deviation (RSD)			**3.8**
F/CZ10/GR02/05C/BW/1R	Bottled Wine	0.01	101.47
F/CZ10/GR02/05C/BW/2R	Bottled Wine	0.1	105.97
Overall mean recovery			**103.72**
Overall standard deviation			**3.18**
Overall relative standard deviation (RSD)			**3.1**

(Romanini M. 2011)

A 2.1.1.2 Description of analytical methods for the determination of residues in tomato

A 2.1.1.2.1 Analytical method 1 – determination of zoxamide and its metabolites in tomato fruits and processed commodities (BPL-STUDY-18-000085)

The analytical phase of the following studies was performed according to method BPL-STUDY-18-000085. The method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4, for the determination of zoxamide (as sum of R and S isomers), (R)-zoxamide, (S)-zoxamide, metabolites RH-141452, RH-150721 (as sum of R and S isomers), (R)-RH-150721, (S)-RH-150721, RH-129151 (as sum of R and S isomers), (R)-RH-129151, (S)- RH-129151, RH-24549, RH-141288 (as sum of R and S isomers), (R)-RH-141288 and (S)-RH-141288 in Raw Agricultural Commodity tomatoes (fruits) and/or processed samples (peeled tomatoes, juice, puree) and is presented under KCP 5.2 as post-authorisation method. The analytical method principle is based on the QuEChERS-method using HPLC-MS/MS.

During the course of the following supervised residue trials procedural recoveries were performed and are presented.

A 2.1.1.2.1.1 Method validation

Comments of zRMS:	The studies 5.1/16, 17 are zoxamide confirmatory studies currently being evaluated by RMS (Latvia). It is also important that these trials are for SEU and not participate/necessary in this authorisation request.

Reference:	**KCP 5.1/16**
Report	Longhi, D., 2020: Determination of (R) and (S) zoxamide residues and its metabolites RH-150721, RH-129151, RH-141452, RH-141288, RH-24549 in raw agricultural commodity of industrial tomato and its processed products (juice, puree and peeled tomatoes) following five applications of formulated product Zoxium 240 SC (Sponsor code GWN-9790 EU) in open field (South Europe – 4 trials year 2018) Gowan Crop Protection Ltd. UK LabAnalysis, Italy, Report No. BPL-STUDY-18-000014, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/17**

Report	Longhi, D., 2020: Determination of zoxamide and its metabolites in raw agricultural commodity of industrial tomato in open field following five applications of the formulated product GWN 9790 EU (South Europe - 4 trials year 2019) Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-19-000059, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical phase of the studies was performed according to method BPL-STUDY-18-000085. The method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4, for the determination of zoxamide (as sum of R and S isomers), (R)-zoxamide, (S)-zoxamide, metabolites RH-141452, RH-150721 (as sum of R and S isomers), (R)-RH-150721, (S)-RH-150721, RH-129151 (as sum of R and S isomers), (R)-RH-129151, (S)- RH-129151, RH-24549, RH-141288 (as sum of R and S isomers), (R)-RH-141288 and (S)-RH-141288 in Raw Agricultural Commodity tomatoes (fruits) and/or processed samples (peeled tomatoes, juice, puree) and is presented under KCP 5.2 as post-authorisation method. During the course of the supervised residue trials procedural recoveries were performed and are presented.

Aliquots of the samples were weighed and extracted twice with an extraction mixture of water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 extracted fractions were pooled and brought up to a final volume of 40 mL (20 mL for potato flakes and potato chips) using the same solvent mixture. After centrifugation, the extracts were transferred into 2 mL glass HPLC vials for final determination with HPLC-MS techniques.

Since the metabolite RH-141452 is known to form conjugates with matrix components, an additional alkaline hydrolysis step was carried out to break the conjugates and release them in their free forms in order to determine the total amount of the analytes (in addition to their already free amounts available in the commodities). For this purpose, a second sample aliquot was treated with an adequate amount of a mixture of acetonitrile/NaOH and hydrolysed at 40°C for 30'. The sample pellet, after neutralization and removal of the liquid phase, was extracted a second time with water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 liquid phases were pooled and brought up to a final volume. They were measured with the analytical method detailed in study BPL-STUDY-18-000085.

For metabolite RH-129151 the correlation between the absolute configuration of the enantiomer (R) or (S) and the corresponding chromatographic peaks was not available; therefore, the first eluted peak was assigned as RH-129151 (A) and the second eluted peak as RH-129151 (B).

Three chromatographic methods were used:

- Method A, for zoxamide and metabolites RH-150721 and RH-141288, by chiral chromatography
- Method B, for metabolite RH-129151, by chiral chromatography
- Method C, for metabolites RH-141452 and RH-24549, by not chiral chromatography

By methods A and B the fraction of the two enantiomers R and S in the found residues were determined.

The analytes were determined with instrumental methods A, B and C, detailed in the separate study summary of BPL-STUDY-18-000085.

Results and discussions

During the course of the supervised residue trials procedural recoveries were measured as follows :

Table A 14: Recovery results of tomatoes (fruits) and processed commodities (BPL-STUDY-18-000014)

Analyte	Recovery (%)	
	LOQ	10xLOQ
Industrial tomato		
Not hydrolysed samples		
(R)-Zoxamide	80.4	105.3
(S)-Zoxamide	84.0	100.7
(R)-RH-150721	77.1	106.2
(S)-RH-150721	76.5	109.5
(R)-RH-141288	91.1	89.3
(S)-RH-141288	104.5	70.6
RH-129151 (A)	106.7	94.5
RH-129151 (B)	105.8	92.5
RH-24549	90.51	93.21
RH-141452	93.23	101.53
	Hydrolysed samples	
RH-141452	83.16	90.09
Tomato juice		
Not hydrolysed samples		
(R)-Zoxamide	86.9	96.8
(S)-Zoxamide	108.8	92.0
(R)-RH-150721	108.8	94.5
(S)-RH-150721	103.1	95.0
(R)-RH-141288	76.5	108.8
(S)-RH-141288	106.7	95.0
RH-129151 (A)	109.8	94.0
RH-129151 (B)	109.4	93.8
RH-24549	104.99	103.3
RH-141452	100.42	102.9
Hydrolysed samples		
RH-141452	89.97	88.66
Tomato puree		
Not hydrolysed samples		
(R)-Zoxamide	93.9	96.2
(S)-Zoxamide	95.2	94.2

(R)-RH-150721	99.3	95.2
(S)-RH-150721	101.0	97.0
(R)-RH-141288	95.6	97.7
(S)-RH-141288	95.8	98.2
RH-129151 (A)	101.6	88.7
RH-129151 (B)	99.2	85.4
RH-24549	97.43	100.65
RH-141452	93.33	109.24
Hydrolysed samples		
RH-141452	101.00	85.19
Peeled tomato		
Not hydrolysed samples		
(R)-Zoxamide	89.7	94.3
(S)-Zoxamide	86.0	96.6
(R)-RH-150721	89.4	94.3
(S)-RH-150721	82.3	92.8
(R)-RH-141288	88.9	70.1
(S)-RH-141288	106.1	70.2
RH-129151 (A)	109.4	91.7
RH-129151 (B)	107.7	90.3
RH-24549	101.60	105.46
RH-141452	109.40	108.79
	Hydrolysed samples	
RH-141452	78.34	98.46

Table A 15: Recovery results of tomato fruits (BPL-STUDY-19-000059)

Analyte	**Recovery (%)**			
	LOQ	**100 x LOQ**	**LOQ**	**100 x LOQ**
Tomato				
Not hydrolysed samples				
Zoxamide (R)	97.5	95.5	117.9*	94.2
Zoxamide (S)	92.4	95.3	120.4*	87.5
RH-141288 (R)	77.8	88.3	116.7*	100.9
RH-141288 (S)	74.2	94.0	120.2*	99.0
RH-150721 (R)	97.1	96.4	113.1*	94.4
RH-150721 (S)	97.6	96.6	112.7*	96.4
RH-129151 (A)	92.1	78.3	103.7	82.5
RH-129151 (B)	78.0	78.9	102.1	81.7
RH-24549	99.11	95.92	80.23	95.55

RH-141452	91.98	99.25	74.08	95.05
Hydrolysed samples				
RH-141452	89.94	79.96	79.76	91.26

* These values are above the limit of 70-110% obtained in the validation (ref. BPL-STUDY-19-000085)

Storage stability of sample extracts

Final sample extracts were analysed as detailed below:

Study no. -	**Residue**	**Max. storage period**
BPL-STUDY-18-000014	Zoxamide, RH-150721, RH-141288, RH-141452, RH-24549	3 days
BPL-STUDY-19-000059	Zoxamide, RH-150721, RH-141288, RH-141452, RH-24549	3 days
	RH-129151	< 24 hours

* No storage stability performed.

The stability of the analytes in the final extracts kept at 4°C for 3 days was successfully verified for all the analytes except RH-129151 in the GLP study no. BPL-STUDY-18-000085. For RH-129151 the stability of the residue in the sample extract over a period of 2 days has been successfully demonstrated by Longhi (2021) in study GLP-STUDY-20-77.

Freezer storage of samples

The maximal storage interval between sampling and analysis was:

Study no.	Residue	Commodity	Max. storage of samples
BPL-STUDY-18-000014	Zoxamide, RH-150721, RH-141288, RH-129151, RH-141452, RH-24549	Industrial tomato, juice, puree, peeled tomato	Industrial tomato: 538 days Tomato juice: 523-529 days Tomato puree: 529 days Peeled tomato: 532 days
BPL-STUDY-19-000059	Zoxamide, RH-150721, RH-141288, RH-129151, RH-141452, RH-24549	Industrial tomato	31 days

Conclusion

The recovery results (mainly) comply with the acceptance criterium of SANCO 825/00, which demands a mean recovery for each matrix and at each fortification level in the range of 70–120%. Therefore, the method BPL-STUDY-18-000085 permits the determination of residues of zoxamide and its metabolites in tomato fruits and its processed fractions with satisfactory accuracy and precision. In addition, the method is regarded as highly specific, using Multi Reaction Monitoring (MRM) with two mass transitions.

The limit of quantification (LOQ) was set at 0.01 mg/kg for zoxamide, RH-150721, RH-129151 and RH-141288 (sum of enantiomers), RH-24549, free RH-141452 and total RH-141452, and 0.005 mg/kg for single isomers. The limit of determination (LOD) was given as 0.003 mg/kg for RH-150721, RH-129151 and RH-141288 (sum of enantiomers) and RH-24549, free RH-141452 and total RH-141452 and 0.0015 mg/kg for single isomers.

(Longhi D. 2020a,b)

A 2.1.1.2.2 Analytical method 2 – determination of zoxamide and benalaxyl-m in tomato fruits and processed commodities (15 F CL GW P/A)

A 2.1.1.2.2.1 Method validation

Comments of zRMS:	The method is accepted. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/18**
Report	Tetuan, B., 2016: Determination of residues at harvest of zoxamide, benalaxyl-m and cymoxanil in tomato, following three broadcast applications of GWN-10392, GWN-9823 and IR6141-copper oxychloride-copper hydroxide 5-15-15 WG under greenhouse conditions and determination of residues at harvest of zoxamide and benalaxyl-m in industry tomato and its processed products (canned tomatoes, puree and juice), following three broadcast applications of GWN-10392, under open field conditions - South Europe - season 2015 Gowan Comercio Internacional e Servicos Limitada, Portugal Promovert Crop Services SL, Spain, Report No. 15 F CL GW P/A, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	Deviation to analytical lab SOP: the weighing of benalaxyl-m for the preparation of the 1 g/L stock solution was less than 10 mg (9.82 mg). This deviation was regarded to have no impact on the results of the study since the balance allows to weigh less 2 mg. Deviation to the study plan: analyses and an analytical phase-based inspection were performed before the finalisation of a study plan amendment – there was no impact on the results as the first daily sample set was not validated. However, the analytical phase-based inspection performed on the 31st December 2015 was kept as the analytical method was the same for the following samples sets.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Residues of benalaxyl-m, cymoxanil and zoxamide in tomato fruits and processed commodities (canned tomatoes, tomato puree and tomato juice) were analysed at Fredon Pays de la Loire / GIRPA in France under report no. B15G-P2-BCZ-01 with a method validated according to SANCO/3029/99 rev. 4 under report no. B15G-P2-BCZ-01. The analytical method principle was based on the method European Committee for Standardisation (CEN): EN 15662:2009-02. "Foods of plant origin – Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE – QuEChERS-method" using HPLC-MS/MS.

Accurately weigh 10 g of ground laboratory sample into a 50 mL polypropylene centrifuge tube. Extract before defrosting. For recoveries, fortify the sample with the appropriate spiking standards solutions using a pipette. Using a measuring cylinder, add about 10 mL of acetonitrile (HPLC quality). Shake manually and vigorously during 1-minute. Transfer the crude extract into a 50 mL QuEChERS tube containing a $MgSO_4$/NaCl/salt tampon. Immediately shake manually and vigorously for 1 minute. Centrifuge for 5

minutes at 4000 rpm. Use an automatic pipette to fill a 6 mL of the organic phase into a 15 mL QuEChERS tube containing 900 mg of $MgSO_4$ and 150 mg of PSA. Shake manually and vigorously during 1 minute. Centrifuge for 5 minutes at 4000 rpm. Dilute twice the clear extract into ultra-pure water. Two mass transitions were monitored for benalaxyl-m, cymoxanil and zoxamide. The limit of quantification (LOQ) was set at 0.01 mg/kg per analyte.

The analytical method used was validated on tomato fruits within this analytical phase by 10 spiked samples, 5 recovery experiments fortified at the LOQ level, 5 recovery experiments fortified at ten times the LOQ level, 2 control samples and a reagent blank.

Equipment:

Instrument: 5500 QTRAP, Autosampler Exsigent, PAL HTC-xt, Pump LC Shimadzu LC-20AD XR
Column: C_{18} Hydro RP (100 mm x 3 mm ID x 2.5 µm PD)
Mobile phase: A: Ultra-pure water / glacial acetic acid (100/0.1) (v/v) + 5 mM ammonium acetate
B: Methanol / glacial acetic 100 : 0.1 (v/v) + 5 mM ammonium acetate

Time table (min)	A (%)	B (%)
0.0	100	0
0.1	100	0
4.0	0	100
6.4	0	100
6.5	100	0
8.0	100	0

Flow rate: 0.7 mL/min
Column temp.: 60°C
Injection volume: 20 µL
Retention time: ~ 4.9 min for benalaxyl-M
~ 3.5 min for cymoxanil
~ 4.8 min for zoxamide
Ionisation: MRM (Positive Multiple reaction Monitoring)
Ion mode
Benalaxyl-m: m/z 326 to m/z 148 (quantification)
m/z 326 to m/z 208 (qualification)
Cymoxanil: m/z 199 to m/z 128 (quantification)
m/z 199 to m/z 111 (qualification)
Zoxamide: m/z 336 to m/z 187 (quantification)
m/z 336 to m/z 159 (qualification)

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 16: Recovery results of benalaxyl-m, cymoxanil and zoxamide

Specimen	Reference items	Level spiked (mg/kg)	Mean recovery rate (%)	RSD (%)	Number of recovery rates (n)
Tomato fruits	Benalaxyl-m	0.010	109	4	5
		0.100	109	8	5
		Overall	109	6	10

Specimen	Reference items	Level spiked (mg/kg)	Mean recovery rate (%)	RSD (%)	Number of recovery rates (n)
	Cymoxanil	0.010	104	4	5
		0.100	106	6	5
		Overall	105	5	10
	Zoxamide	0.010	104	5	5
		0.100	104	9	5
		Overall	104	7	10

Accuracy and repeatability/precision

Mean recoveries per fortification level were around 100 % with % RSD values less than 20%, the method therefore fulfils the requirements for residue analytical methods. Thus, demonstrating a satisfying accuracy and precision of the method.

Linearity

Linearity of the calibration curve was demonstrated between 1.5 to 20 µg/L for benalaxyl-m and zoxamide and 1.5 to 200 µg/L for cymoxanil over at least 5 measurement points (corresponding to 0.003 – 0.40 mg a.s./kg for each analyte). The correlation coefficients were typically higher than 0.99.
The analytical calibration spans over a range including the lowest and highest nominal concentration of the reference item in the analytical solutions ± at least 20%.

Limit of quantification (LOQ)

The limit of quantification of the method was set at the lowest validated level where a mean recovery within the range 70-110% with a RSD less or equal to 20% could be obtained. The LOQ for benalaxyl-m, cymoxanil, zoxamide is 0.01 mg/kg.

Matrix effects

Any matrix effects were compensated using matrix-matched standard. Analysis of control specimens of tomato fruits yielded no residues of benalaxyl-m, cymoxanil or zoxamide above 30% of the limit of quantification, indicating that no interference was present at the retention time of these reference items.

Specificity

The specificity was checked by analysis of at least one untreated specimen (two repetitions) and at least one reagent blank. Interferences due the substrate were less than 30% of the limit of quantification.

The method using LC-MS/MS is regarded as specific, monitoring two ion mass transitions.

Stability of sample extracts

Final sample extracts were analysed within 24 hours.

Storage stability of frozen samples

The maximal storage interval between sampling and analysis was 153 days.

Table A 17: Characteristics for the analytical method validation for the determination of benalaxyl-m, cymoxanil and zoxamide residues in tomatoes

	Benalaxyl-m	**Cymoxanil**	**Zoxamide**
Specificity	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standardcalibration, 5 points calibration; r^2>0.99; linear Regression Model y = 1003528,51x + 186828,04 Individual calibration data and calibration line equation presented in the study report	Matrix matched standardcalibration, 8 point calibration; r^2>0.99; linear. Regression Model y = 66329,88x + 1929,67 Individual calibration data and calibration line equation presented in the study report	Matrix matched standardcalibration, 5 points calibration; r^2>0.99; linear Regression Model y = 291380,50x + 45430,50 Individual calibration data and calibration line equation presented in the study report
Calibration range	**1.5 to 20 µg/L (corresponding to 0.003 – 0.40 mg a.s./kg)**	**1.5 to 200 µg/L (corresponding to 0.003 – 0.40 mg a.s./kg)**	**1.5 to 20 µg/L (corresponding to 0.003 – 0.40 mg a.s./kg)**
Assessment of matrix effects is presented	Yes	Yes	yes
Limit of quantification (LOQ)	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg

The following figures show chromatograms

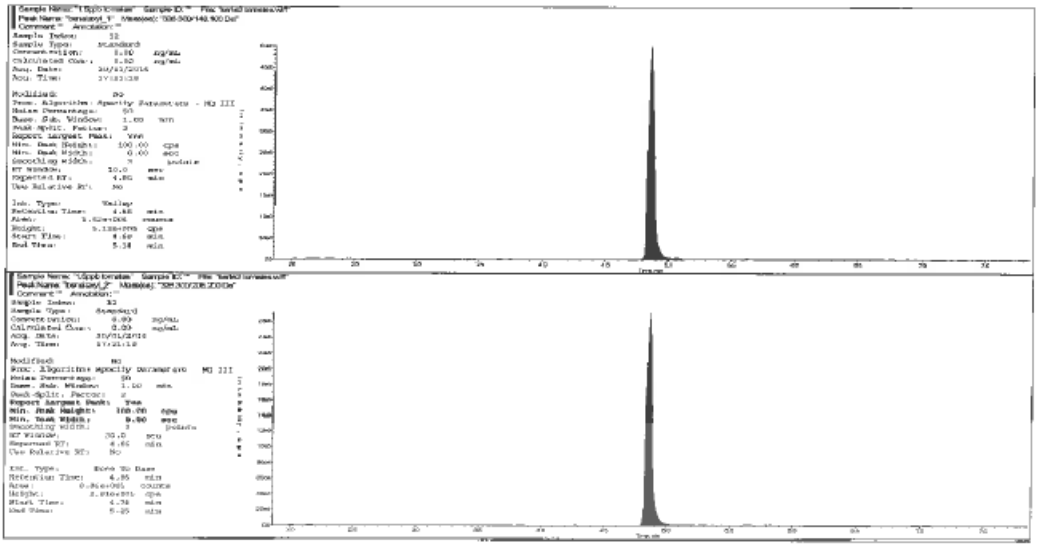


Figure A 4: Typical chromatogram of benalaxyl-m

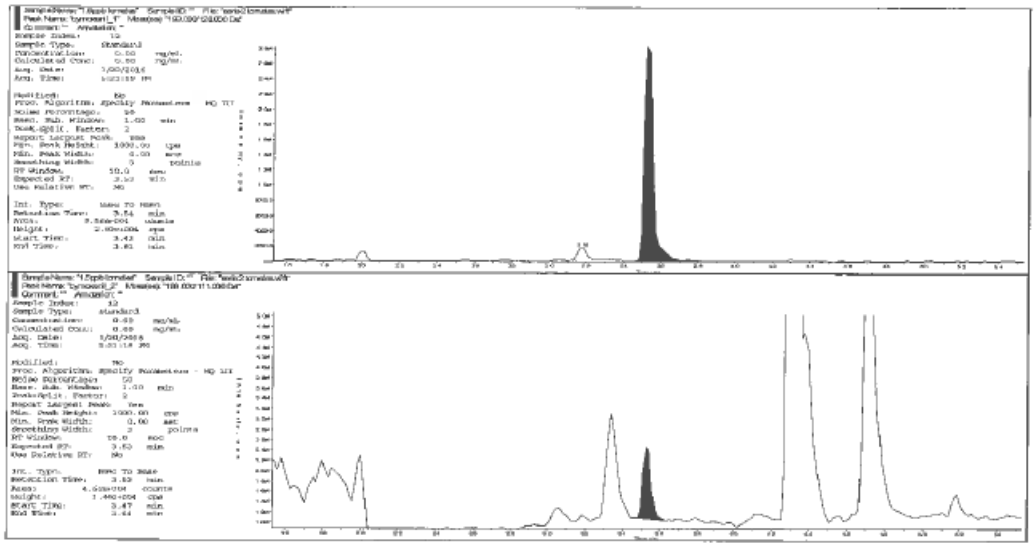


Figure A 5: Typical chromatogram of cymoxanil

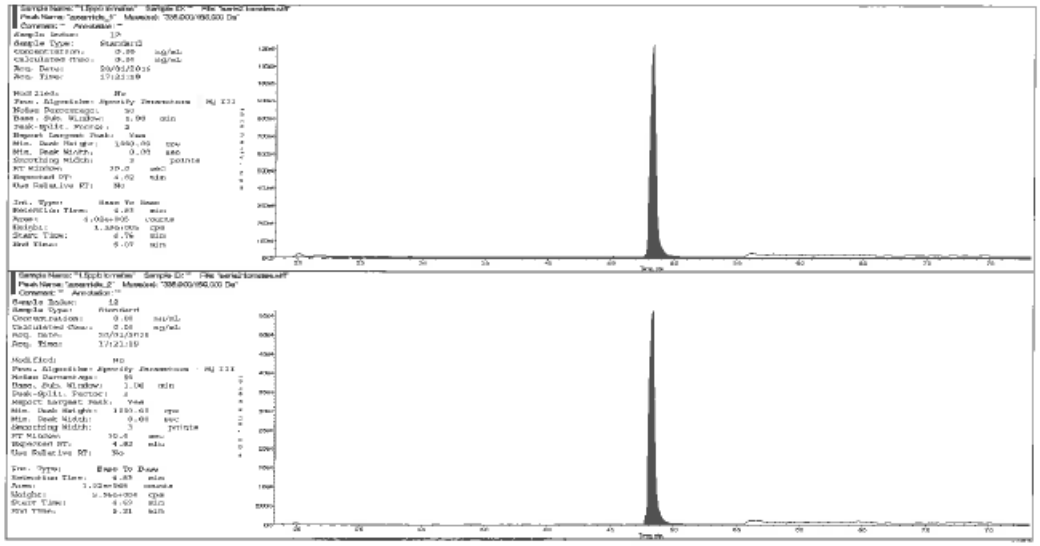


Figure A 6: Typical chromatogram of zoxamide

Conclusion

An LC-MS/MS method (QuEChERS-method) for the determination of zoxamide, cymoxanil and benalaxyl-m in tomato fruits and processed commodities (canned tomatoes, tomato puree and tomato juice) has been successfully validated according to SANCO/3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

(Tetuan B. 2016)

A 2.1.1.2.3 Analytical method 3 – determination of zoxamide and cymoxanil in tomato fruits and processed commodities (CREG2118)

A 2.1.1.2.3.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/19**
Report	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity tomato (fruit, juice, puree and canned) following five applications of HARPON WG (Cymoxanil 33% + zoxamide 33% WG) - Four trials, Italy 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2118, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANC0/825/00 rev.7 (2004)
Deviations:	No
Acceptability:	Yes

Materials and methods

For cymoxanil the sample was extracted by Ultra-Turrax with ethyl acetate, purified by liquid-liquid partition and analysed by gas chromatography with a Nitrogen Phosphorus Detector (GC/NPD). Zoxamide was determined using an internal analytical method that consisted in a solvent extraction by Ultraturrax (bunch samples) or by shaking (processed samples). The extract containing the active ingredient was cleaned up by liquid-liquid partition and by SPE chromatography, then analysed by gas chromatography equipped with an ECD. The Limit of Quantification (LOQ) was 0.05 mg/kg for cymoxanil on tomato samples and 0.01 mg/kg on processed samples; for zoxamide the LOQ was 0.01 mg/kg for all matrices. Limit of Detection (LOD) was 0.0199 mg/kg for tomato samples and 0.0249 mg/kg for processed samples (juice, puree and canned) for cymoxanil, and 0.0051 for zoxamide in all matrices.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.4980 µg/mL to 2.490 µg/mL (cymoxanil) and 0.005130 µg/mL to 0.1028 µg/mL (zoxamide). The linear correlation was calculated for each analyte in wine matrix:

- Cymoxanil
  r2 =0.999909 Y= 0.081470X+0.089616 (n= 6)
- Zoxamide
  r2 = 0.991140 Y= 0.000011X+0.000929 (n= 6)

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO01/01C/1R	Tomato / Fruit	0.01	97.76
I/CZ10/TO01/01C/2R	Tomato / Fruit	0.1	94.55
I/CZ10/TO02/03C/1R	Tomato / Fruit	0.01	105.99
I/CZ10/TO02/03C/2R	Tomato / Fruit	0.1	100.16
I/CZ10/TO03/07C/2R	Tomato / Fruit	0.1	109.55
I/CZ10/TO03/07C/3R	Tomato / Fruit	0.01	107.21
I/CZ10/TO03/07C/4R	Tomato / Fruit	0.01	102.34
I/CZ10/TO04/09C/1R	Tomato / Fruit	0.01	108.19
I/CZ10/TO04/09C/2R	Tomato / Fruit	0.1	97.17
I/CZ10/TO04/09C/3R	Tomato / Fruit	0.5	84.68
I/CZ10/TO04/09C/4R	Tomato / Fruit	0.5	81.52
Overall mean recovery			**99.01**
Overall standard deviation			**9.27**
Overall relative standard deviation (RSD)			**9.4**

Table 2: Recovery test result on tomato / fruit samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO02/05C/JU/1R	Tomato / Juice	0.01	97.47
I/CZ10/TO02/05C/JU/2R	Tomato / Juice	0.1	86.55
I/CZ10/TO02/05C/PU/1R	Tomato / Puree	0.01	81.87
I/CZ10/TO02/05C/PU/2R	Tomato / Pureee	0.1	93.66
I/CZ10/TO02/05C/CA/1R	Tomato / Canned	0.01	108.19
I/CZ10/TO02/05C/CA/2R	Tomato / Canned	0.1	93.08
Overall mean recovery			**93.47**
Overall standard deviation			**9.11**
Overall relative standard deviation (RSD)			**9.8**

Table 3: Recovery test result on tomato / processed samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO01/01C/1R	Tomato / Fruit	0.01	97.76
I/CZ10/TO02/03C/1R	Tomato / Fruit	0.01	105.99
I/CZ10/TO03/07C/3R	Tomato / Fruit	0.01	107.21
I/CZ10/TO03/07C/4R	Tomato / Fruit	0.01	102.34
I/CZ10/TO04/09C/1R	Tomato / Fruit	0.01	108.19
Overall mean recovery			**104.30**
Overall standard deviation			**4.27**
Overall relative standard deviation (RSD)			**4.1**

Table 4: Recovery test result on tomato / fruit samples at LOQ (0.01 mg/kg) spiking level

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO02/05C/JU/1R	Tomato / Juice	0.01	97.47
I/CZ10/TO02/05C/PU/1R	Tomato / Puree	0.01	81.87
I/CZ10/TO02/05C/CA/1R	Tomato / Canned	0.01	108.19
Overall mean recovery			**95.84**
Overall standard deviation			**13.24**
Overall relative standard deviation (RSD)			**13.8**

Table 5: Recovery test result on tomato / processed samples at LOQ (0.01 mg/kg) spiking level

(Romanini M. 2011)

A 2.1.1.2.4 Analytical method 4 –determination of zoxamide in tomato fruit, puree and paste (34-99-111)

A 2.1.1.2.4.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/20**
Report	Devine, H.C., 2008: Residues of Mancozeb and zoxamide in field and protected tomatoes at intervals and at harvest following multiple applications of Electis, Northern France and The United Kingdom – 2006 Dow Agrosciences Ltd., UK CEM Analytical Services Ltd (CEMAS), UK, Report No. CEMS-2967, GLP, Not published
Guideline(s):	ENV/JM/MONO (99)22 ENV/MC/CHEM (98)17 ENV/JM/MONO (2002)/9
Deviations:	No
Acceptability:	Yes

Materials and methods

The analytical method used for analysis of zoxamide in the samples from this study was the procedure detailed in Rohm and Hass Technical report No. 34-99-111 with a Limit of Quantification (LOQ) of 0.01 mg/kg

Residues of zoxamide in untreated samples were not detected or <LOQ with the exception of the sample after the final application from trial CEMS-2967E which had a residue of 0.01 mg/kg; residues in treated samples are summarised below.

The samples were kept cool and in the dark from the time of collection and place in freezers working at -18°C, within approximately 6 hours of sampling.

Samples were weighed into a centrifuge bottle and extracted with acetonitrile. The acetonitrile was concentrated by rotary evaporation. This extract was cleaned-up using liquid-liquid partitioning into ethyl acetate before being dried by rotary evaporation and reconstituted in hexane. The extract was then purified using a carbon solid phase extraction (SPE) cartridge. The eluate was dried by rotary evaporation and re-constituted in hexane before analysis by Gas Chromatography with Electron Capture Detection (GC-ECD).

Results and discussions

Table A 18: Recoveries of zoxamide from tomato

Substrate	Fortification Level (mg/kg)	Recovery (%)
Tomato fruit	0.01	95
	0.10	85
	0.01	92

	0.10	117
	0.01	93
	0.10	87
	0.01	78
	0.10	74
	Mean (%)	**90**

(Devine H.C. 2008)

A 2.1.1.3 Description of analytical methods for the determination of residues in potatoes

A 2.1.1.3.1 Analytical method 1 – determination of zoxamide with QuEChERS method in potato tubers

A 2.1.1.3.1.1 Method validation

Comments of zRMS:	The studies 5.1/21, 22 were already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/21**
Report	Tetuan, B., 2011: Determination of residues at harvest in potatoes, following six broadcast applications of Harpon WG, under field conditions - Northern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/A, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/22**
Report	Tetuan, B., 2011: Determination of residues at harvest in potatoes, following five broadcast applications of HARPON WG, under field conditions - Southern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/B, PROMO/ZOX-CYM/10.01, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes

Acceptability: Yes

Residue concentrations were determined by LC/MS/MS analysis using method based on GIRPA (GIR/MET/CYMOXANI/04V1 and GIR/MET/ZOXAMIDE/02V1 Analytical method was based on the multiresidue QuEChERS method (Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - European method NF EN 15662) and on the Dow AgroSciences GRM 02.07.R1 method (Residue analysis of zoxamide in grapes, potatoes and tomatoes using GC-MS detection – 09/12/2002 – Ana Cristina Pinheiro and Roberto De Vito).

Residues of cymoxanil and zoxamide are extracted from potatoes in frozen condition with the help of an acetonitrile/2% potassium bicarbonate aqueous solution (80/20, v/v) mixture. After addition of magnesium sulfate, sodium chloride and buffering citrate salts, the mixture was shaken intensively and centrifuged for phase separation. An aliquot of the organic phase was cleaned-up by dispersive solid phase extraction (D-SPE). **For each reference item,** the determination was performed by liquid chromatography with detection by mass spectrometric in tandem (LC-MS/MS) **with two different mass transitions, one for quantification and one for qualification.**

Results

Specificity

For each reference item, the specificity of the method has been demonstrated; interferences due to the substrate were less than 30% of the limit of quantification.
The reagent blank showed that no interference due to the reagents was detected.
The chromatographic method on LC-MS/MS **following two transitions** is highly specific, an additional confirmatory method was not necessary.

Qualitative confirmation was carried out by comparison of the relative abundances of the qualification transition (% relative to quantification transition) in spiked sample extracts, with those in the calibration standards.
Summary of quantification/qualification transitions used:

Summary of quantification/qualification transitions used:

Reference item	*Quantification transition*	*Qualification transition*
Cymoxanil	*199 → 128*	*199 → 111*
Zoxamide	*336 → 187*	*336 → 159*

All the transitions are specific for each reference item. The most sensitive transitions were chosen for quantification.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.004 mg/L to 0.030 mg/L. The linear correlation was calculated for each analyte in potato matrix:

- Cymoxanil
  r2 =0.999966 Y= 10452X+1325.7 (n= 5)
- Zoxamide
  r2 = 1 Y= 34910X+11437 (n= 5)

LOQ = 0.01 mg/kg for both substances.

Repeatability/Accuracy

Specimen	Reference item	Level spiked ($mg.kg^{-1}$)	Recovery rate (%)	Relative standard deviation (%)	Number of recovery rates (n)
Potatoes	Cymoxanil	0.010	101	4	5
		0.100	110	5	5
		all	106	6	10
	Zoxamide	0.010	104	2	5
		0.100	110	5	5
		all	107	5	10

(Tetuan B. 2011)

A 2.1.1.3.2 Analytical method 2 – determination of zoxamide in potato tubers and processed fractions with method BPL-STUDY-18-000085

The analytical phase of the following studies was performed according to method BPL-STUDY-18-000085. The method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4, for the determination of zoxamide (as sum of R and S isomers), (R)-zoxamide, (S)-zoxamide and the metabolites (R)-RH-150721, (S)-RH-150721, (R)-RH-141288 and (S)-RH-141288, (A)-RH-129151 and (B)-RH-129151, RH-141452 and RH-141455 in Raw Agricultural Commodity potatoes (tubers) and/or processed samples (flakes, chips) and is presented under KCP 5.2 as post-authorisation method. The analytical method principle is based on the QuEChERS-method using HPLC-MS/MS.

During the course of the following supervised residue trials procedural recoveries were performed and are presented.

A 2.1.1.3.2.1 Method validation

~~Comments of zRMS:~~	~~For evaluation of 5.1/23, 25 studies please, see Section 7 of the present RR. Study 5.1/24 is zoxamide confirmatory study currently being evaluated by zoxamide RMS (Latvia).~~

Comments of zRMS:	The study is acceptable. Two harvest trials were conducted in NEU. The objective of the study is to determine the magnitude of the residues of zoxamide and metabolite RH-1452 and RH-1455 in raw potatoes (RAC tubers) and processed fractions after 5 applications of GWN 9790 EU. The application rate was 0.75 L/ha, representing 180 g/ha of zoxamide at each application, starting at 36 or 39 DBH, with 8 ± 1 days interval, last application 7 days before harvest. The analysis was performed on the basis of the analytical method described and validated separately from this study (see B5, BPL-STUDY-18-000085). The analytical method was validated in potato following SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4. During the study analysis acceptance criteria for method validations were met, with average recoveries ranging from 70% to 110%. The LOQ for zoxamide and metabolites RH-141452 and RH-141455 was 0.010 mg/kg. No residue of zoxamide and its metabolites RH-141452 and RH-141455 were found above the LOQ in all the specimens (RAC tubers, potato flakes and French fries).

Reference:	**KCP 5.1/23**
Report	Terranegra, A., 2020: Magnitude of residue of zoxamide and metabolite RH-1452 and RH-1455 in potatoes (RAC tubers) and processed fractions, following 5 applications of GWN 9790 EU in two trials (2 HS), Northern Europe (France and Poland) – 2017 – amended Final Report GOWAN Crop Protection Ltd., UK Staphyt Italia S.r.l., Italy, Report No. ATA-18-30694, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4 (2000) SANCO/825/00 rev.8.1 (2010)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

And

Comments of zRMS:	Not relevant. Submitted as support for B7 and for SEU – therefore not evaluated.

Reference:	**KCP 5.1/24**
Report	Pandolfi, A., 2020: Determination of the residues of zoxamide (R), (S) and sum and its metabolites in raw agricultural commodity potato (tubers) and its processed fractions (chips, baked/cooked, fried and flakes) following five applications of Zoxium 240 SC (sponsor code GWN-9790 EU) in open field

	condition (Italy - Southern Europe - 2 trials year 2018) Gowan Grop Protection Ltd., UK Res Agraria S.r.l., Italy, Report No. RA 18 051 BPL GW, BPL-STUDY-19-000025, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000).
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Comments of zRMS:	The study is acceptable. The validation parameters were within the required range. In HPLC-MS/MS method transitions for (R) and (S) zoxamide were monitored. The sufficient stability was demonstrated. The method employed was validated according to the SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 guidelines in the BPL-STUDY-18-000085.

Reference:	**KCP 5.1/25**
Report	Sala, A., 2021: Interim report – storage stability of zoxamide residues under frozen conditions (-18°C) in potato tubers, potato flakes and fried potatoes Gowan Crop Protection Ltd, UK LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-18-000047, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	The recovery check results for RH-150721 (R) in the samples 18/47/FP/89R and 18/47/FP/90R (67.4% and 62.2%) were below the acceptability range (70% - 110%). The recovery check results for RH-141452 and RH-141455 in the samples 18/47/PF/56R and 18/47/PF/57R (61.6% and 54.4%) were below the acceptability range (70% - 110%). However, retain samples were extracted along with recovery checks prepared with leftover starting material (BPL-SMPL-18-000421). The recovery check results for RH-141455 in the samples 18/47/PF/63R and 18/47/PF/64R (52.5% and 53.6%) were below to the acceptability range (70-110%). However, the results obtained from the samples 18/47/PF/61T and 18/47/PF/62T were close to the recovery check ones and, if corrected, the results obtained are close to 100%. Due to unexpected reason, the calibration point at level 4 (tuber L4) for the analyte RH-141455 in the calibration curve, had a response higher than expected. However, the calibration range was not altered and 4 points were enough for a correct interpolation. This deviation was solved with no impact on the study. The recovery check results for RH-141455 in the samples 18/47/PO/30R and 18/47/PO/31R (128.1% and 125.5%) were above the acceptability range

	(70% - 110%). However, the above deviations were regarded as not relevant for the integrity of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analyses were carried out under analytical phase report no. BPL-STUDY-19-000065 and BPL-STUDY-19-000025 using a HPLC-MS/MS method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 guidelines, in the study coded BPL-STUDY-18-000085: "Validation of an analytical method to determine zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities", Test Facility: LabAnalysis s.r.l., Study Director: Alberto Sala.

The method consists in an extraction using a mixture of water/acetonitrile/methanol. After addition of same solvent mixture, the mixture was centrifuged for separation phase. The determination was performed with HPLC-MS techniques.

The residue of zoxamide and its metabolite containing a chiral centre (RH-150721) were expressed as sum of the 2 enantiomers as well as single enantiomer (R) and single enantiomer (S).

The metabolite RH-141452 and RH-141455 are known to form conjugates with the matrix: in order to determine the total amount of the analyte (in addition to the free form) a hydrolysis step was carried out in order to break the conjugates and to release the free analyte.

Analytical aliquots of the samples were weighed and extracted twice with an extraction mixture of water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 extracted fractions were pooled and brought up to a final volume of 40 mL (20 mL for potato flakes) using the same solvent mixture. After centrifugation the extract was transferred in a 2 mL glass HPLC vial for final determination with HPLC-MS techniques.

For the determination of the total amount of the metabolite RH-141452 and RH-141455, that are known to form matrix conjugated metabolites, an alkaline hydrolysis step was required to release them in their free form. For this purpose, a second sample aliquot was treated with an adequate amount of a mixture of acetonitrile/NaOH and hydrolysed at 40°C for 30'. The sample pellet, after neutralization and removal of the liquid phase, was extracted once more with water/acetonitrile/methanol (20/40/40 v/v/v) containing 0.2% of formic acid. The 2 liquid phases were pooled and brought up to a final volume.

Limit of quantification (LOQ) achieved was 0.010 mg/kg for zoxamide (expressed as sum), for metabolites RH-141452 and RH-141455.

Limit of determination (LOD) was found to be 0.003 mg/kg.

Two chromatographic methods were used:

Method A, for zoxamide and metabolites RH-150721 and RH-141288, by chiral chromatography.

Method D, for zoxamide and metabolites RH-141452 and RH-141455by non-chiral analytes.

The analytes were determined with instrumental methods A and D (for the determination of chiral and non-chiral analytes) of the method and the validation results is given in the study report and the separate study summary of BPL-STUDY-18-000085

Table A 19: R_f values and MRM monitored transitions – instrumental method A

MRM transitions monitored							
Analyte	**Retention time (min)**	**Detection**	**Precursor ion (m/z)**	**Product ion (m/z)**	**Dwell (mS)**	**Fragmentor (V)**	**Collision energy (V)**
(R)-Zoxamide	16.9	Primary	338	188.9	50	125	25
		Confirmatory	336	186.9			
(S)-Zoxamide	15.4	Primary	338	188.9	50	125	25
		Confirmatory	336	186.9			
(R)-RH-150721	13.1	Primary	320	188.9	200	125	21
		Confirmatory	318	186.9			
(S)-RH-150721	16.1	Primary	320	188.9	200	125	21

Table A 20: R_f values and MRM monitored transitions – instrumental method D

Ions monitored							
Analyte	**Retention time approx. (min.)**	**Time segment scan (min.)**	**Detection**	**Parent ions (m/z)**	**Fragment ions (m/z)**	**Collision energy (eV)**	**Polarity**
RH-141455	9.29	0.00 – 9.60	Primary	232.9414 (SIM)		/	Negative
			Confirmatory	232.9414	188.9516	20	
RH-141452	10.28	9.61 – 11.70	Primary	218.9621 (SIM)		/	
			Confirmatory	218.9621	144.9617	20	

Results and discussions

Recovery findings

Table A 21: Recovery results in potatoes (ATA-18-30694)

Analyte	**Potato tuber**	
	19/65/PO/RC1/NH	**19/65/PO/RC2/NH**
Not hydrolysed		
Zoxamide (R)	93.6	100.4
Zoxamide (S)	99.7	98.6
RH-141452	85.2	99.7
RH-141455	82.9	93.7
Hydrolysed		
RH-141452	79.7	85.8
RH-141455	72.5	64.0
RH-141452	79.7	85.8
	Potato flakes	
	19/65/PF/RC1/NH	**19/65/PF/RC2/NH**
Not hydrolysed		
Zoxamide (R)	103.6	97.0
Zoxamide (S)	108.5	96.8
RH-150721 (R)	108.5	105.6
RH-150721 (S)	95.5	107.3
RH-141452	86.7	94.6
RH-141455	92.8	88.2
Hydrolysed		
RH-141452	72.3	92.5

RH-141455	69.7	70.5
	French fries	
	19/65/FP/RC1/NH	**19/65/FP/RC2/NH**
Not hydrolysed		
Zoxamide (R)	82.8	71.2
Zoxamide (S)	83.0	71.6
RH-150721 (R)	76.8	71.0
RH-150721 (S)	80.2	71.0
RH-141452	75.2	90.2
RH-141455	77.2	88.3
Hydrolysed		
RH-141452	89.7	90.7
RH-141455	87.2	71.0

Table A 22: Recovery results in potato tuber and baked/cooked potato (not hydrolysed) (RA 18 051 BPL GW)

Analyte	**Recovery (%)** (Limit 70-110%)			
	Potato tuber			
	BPL-SMPL-19-000708/NH RC1	**BPL-SMPL-19-000708/NH RC2**	**BPL-SMPL-19-000708/NH RC1 R**	**BPL-SMPL-19-000708/NH RC2 R**
	LOQ	**10xLOQ**	**LOQ**	**10xLOQ**
Zoxamide (R)	70.5	90.3	Not evaluated: the analytical sequence was reanalysed only for RH-141452 and RH-141455	Not evaluated: the analytical sequence was reanalysed only for RH- 141452 and RH-141455
Zoxamide (S)	73.9	95.3		
RH-141452	Not evaluated due to the failure of the analytical sequence (reanalysed)	Not evaluated due to the failure of the analytical sequence (reanalysed)	80.03	101.7
RH-141455			106.3	108.07
	Baked/cooked potato			
	BPL-SMPL-19-000718/NH RC1	**BPL-SMPL-19-000718/NH RC2**	**BPL-SMPL-19-000718/NH RC1 R**	**BPL-SMPL-19-000718/NH RC2 R**
	LOQ	**10xLOQ**	**LOQ**	**10xLOQ**
Zoxamide (R)	70.5	75.7	Not evaluated: the analytical sequence was reanalysed only for RH-141452 and RH-141455	Not evaluated: the analytical sequence was reanalysed only for RH-141452 and RH-141455
Zoxamide (S)	71.4	75.6		
RH-150721 (R)	71.8	71.0		
RH-150721 (S)	71.3	71.2		
RH-141452	Not evaluated due to the failure of the analytical sequence (reanalysed)	Not evaluated due to the failure of the analytical sequence (reanalysed)	71.11	72.71
RH-141455			99.26	72.81

Table A 23: Recovery results in potato chips, potato flakes and fried potato (not hydrolysed) (RA 18 051 BPL GW)

Analyte	**Recovery (%)** (Limit 70-110%)					
	Potato chips		**Potato flakes**		**Fried potato**	
	BPL-SMPL- 19-000717/NH RC1	**BPL-SMPL-19-000717/NH RC2**	**BPL-SMPL-19-000720/NH RC1**	**BPL-SMPL-19-000720/NH RC2**	**BPL-SMPL-19-000719/NH RC1**	**BPL-SMPL-19-000719/NH RC2**

	LOQ	10xLOQ	LOQ	10xLOQ	LOQ	10xLOQ
Zoxamide (R)	96.5	87.6	80.7	99.2	101.4	72.8
Zoxamide (S)	95.0	88.4	74.4	99.8	102.8	72.9
RH-150721 (R)	98.9	97.6	83.2	100.9	104.6	77.1
RH-150721 (S)	102.1	92.7	86.0	99.9	101.7	78.9
RH-141452	104.72	108.41	107.23	99.19	84.62	96.09
RH-141455	78.34	109.28	71.54	89.3	89.61	99.92

Table A 24: Recovery results in potato tuber and baked/cooked potato (hydrolysed) (RA 18 051 BPL GW)

	Recovery (%) (Limit 70-110%)					
Analyte	Potato tuber		Baked/cooked potato			
	BPL-SMPL-19-000708/H RC1	BPL-SMPL-19-000708/H RC2	BPL-SMPL-19-000718/H RC1	BPL-SMPL-19-000718/H RC2	BPL-SMPL-19-000718/H RC1 R	BPL-SMPL-19-000718/H RC2 R
	LOQ	10xLOQ	LOQ	10xLOQ	LOQ	10xLOQ
RH-141452	92.87	91.27	Not evaluated due to the failure of the analytical sequence (reanalysed)	Not evaluated due to the failure of the analytical sequence (reanalysed)	96.76	105.36
RH-141455	92.2	86.75			91.08	100.9

Table A 25: Recovery results in potato chips, potato flakes and fried potato (hydrolysed) (RA 18 051 BPL GW)

	Recovery (%) (Limit 70-110%)					
Analyte	Potato chips		Potato flakes		Fried potato	
	BPL-SMPL-19-000717/H RC1	BPL-SMPL- 19-000717/H RC2	BPL-SMPL- 19-000720/H RC1	BPL-SMPL- 19-000720/H RC2	BPL-SMPL-19-000719/H RC1	BPL-SMPL-19-000719/H RC2
	LOQ	10xLOQ	LOQ	10xLOQ	LOQ	10xLOQ
RH-141452	76.23	86.09	71.57	91.92	72.82	91.84
RH-141455	79.86	109.77	72.82	70.71	94.04	95.58

Table A 26: Results of procedural recovery test of the days (BPL-STUDY-18-000047)

Sample code	Fortification level of recovery test (µg/g)	Potato (tuber) Procedural recovery test of the day - Result (%)				
		Zoxamide sum	(R)-Zoxamide	(S)-Zoxamide	RH-141452	RH-141455
		0 Days (2h after spiking)				
18/47/PO/4R	0.1	97.1	95.4	98.8	96.7	109.8
18/47/PO/5R	0.1	100.2	98.4	101.9	96.0	109.6
	Mean (%)	**98.7**	**96.8**	**100.3**	**96.4**	**109.7**
		3 months				
18/47/PO/9R	0.1	99.2	98.8	99.6	99.2	109.5
18/47/PO/10R	0.1	99.1	98.7	99.5	99.3	109.0
	Mean (%)	**99.2**	**98.7**	**99.6**	**99.2**	**109.3**
		6 months				
18/47/PO/16R	0.1	98.0	97.6	98.4	103.6	106.7
18/47/PO/17R	0.1	96.3	95.5	97.0	104.2	108.2
	Mean (%)	**97.2**	**96.6**	**97.7**	**103.9**	**107.4**
		12 months				

18/47/PO/23R	0.1	101.0	101.4	100.5	109.2	109.8
18/47/PO/24R	0.1	99.1	98.8	99.3	107.9	109.8
	Mean (%)	**100.1**	**100.1**	**99.9**	**108.5**	**109.8**
		18 months				
18/47/PO/30R	0.1	81.7	81.4	81.9	94.1	128.1
18/47/PO/31R	0.1	81.6	80.8	82.4	93.3	125.5
	Mean (%)	**81.7**	**81.1**	**82.2**	**93.7**	**126.8** [2]
		22 months				
18/47/PO/42R	0.1	96.6	95.5	97.6	-	-
18/47/PO/43R	0.1	97.7	97.2	98.1	-	-
	Mean (%)	**97.2**	96.3	97.8	-	-

- Not Analysed
2 : See deviation

Accuracy and repeatability/precision

Accuracy (mean recovery per level) and precision (relative standard deviation % - RSD% per level) for each pair analyte/matrix are reported in the following tables, the results reported are referred to both primary and confirmatory detection (a simultaneous confirmation of the primary detection was carried out by monitoring a second MRM transition or a second-high resolution mass ion for each analyte).

All mean accuracy and precision values found fulfil SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. Guidelines: mean recovery (accuracy) per level in the range 70-110 %, RSD% per level (precision) ≤ 20%.

Linearity

Linearity of response was verified in all analytical runs in the following conditions:
- All calibration curve prepared with matrix matched analytical standard.
- 5 points calibration curves.
- Calibration range: 1-50 µg/L on final extract (exceptions: for wine e grape juice samples, nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flakes samples the lowest concentration was 0.7µg/L corresponding to a concentration on sample of 2.8 µg/kg.
- 3-160 mg/kg on sample
- 30% LOQ – 50 x LOQ
- For analytes with a chiral center: levels referred to the sum of the 2 enantiomers.
- $R^2 > 0.99$.
- Linearity always checked for the 2 MRM (Multiple Reaction Monitoring) transition in case of Triple quadrupole mass analysis or for the 2 high resolution ions in case of HRMS-Orbitrap analysis.

Limit of quantification

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery and an acceptable relative standard deviation are obtained.

- 0.01 mg/kg, for zoxamide sum R+S, RH-150721 sum R+S, RH-141288 sum R+S, RH-129151 sum A+B, RH-24549, Free RH-141452 and Total RH-141452,
- 0.005 mg/kg for (R)-zoxamide, (S)-zoxamide, (R)-RH-150721, (S)-RH-150721, (R)-RH-141288 and (S)-RH-141288, (A)-RH-129151 and (B)-RH-129151.

Limit of detection

The limit of detection (LOD) is the lowest amount that can be detected but not necessarily quantitated as an exact value: the limit of detection was set at 30 % of LOQ.

- 0.03 mg/kg, for zoxamide sum R+S, RH-150721 sum R+S, RH-141288 sum R+S, RH-129151 sum A+B, RH-24549, Free RH-141452 and Total RH-141452,
- 0.0015 mg/kg for (R)-zoxamide, (S)-zoxamide, (R)-RH-150721, (S)-RH-150721, (R)-RH-141288 and (S)-RH-141288, (A)-RH-129151 and (B)-RH-129151.

Matrix effects

All calibration curves, for all matrices/analyte were prepared using matrix matched analytical standards.

Blank sample extracts, prepared with the same procedure applied on spiked samples were used to dilute stock solutions. Using this approach, the matrix effect on all samples analysed was nullified.

Specificity

For all analytes in all matrices analysed the signal in the blank samples (both primary detection and confirmatory detection) resulted always lower than the lowest calibration level injected (corresponding to 30% of the target LOQ) in most cases the signal in the blank samples resulted zero (i.e. no detectable, no quantifiable at analyte retention time).

Stability of Sample Extracts

Final sample extracts were analysed as detailed below:

Study no.	Residue	Max. storage of sample extracts
ATA-18-30694, BPL-STUDY-19-000065	--	24 hours
RA 18 051 BPL GW	--	24 hours

Storage stability of frozen samples

The maximal storage interval between sampling and analysis was:

Study no.	Residue	Commodity	Max. storage of samples
ATA-18-30694, BPL-STUDY-19-000065	Zoxamide, RH-141452, RH-141455, RH-150721	Potato tubers, potato flakes, potato fries	619-638 days
RA 18 051 BPL GW	Zoxamide, RH-141452, RH-141455, RH-150721	Potato tubers, potato chips, baked/cooked potatoes, fried potatoes, potato flakes	600-634 days

Table A 27: Characteristics for the analytical method for the determination of zoxamide residues in potatoes

	Zoxamide, RH-141452, RH-150721, RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix-matched standard calibration. 5-point calibration; r^2 = >0.99; linear Calibration data and calibraition line equation presented in the study report
Calibration range	1-50 µg/L on final extract (exceptions: for wine e grape juice samples, nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flakes samples the lowest concentration was 0.7µg/L corresponding to a concentration on sample of 2.8 µg/kg, 3-160 mg/kg on sample

	Zoxamide, RH-141452, RH-150721, RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	LOQ: - 0.01 mg/kg for sum - 0.005 mg/kg for analytes with a chiral centre (for each enantiomer) LOD: - 0.003 mg/kg for sum - 0.0015 mg/kg for analytes with a chiral centre (for each enantiomer)

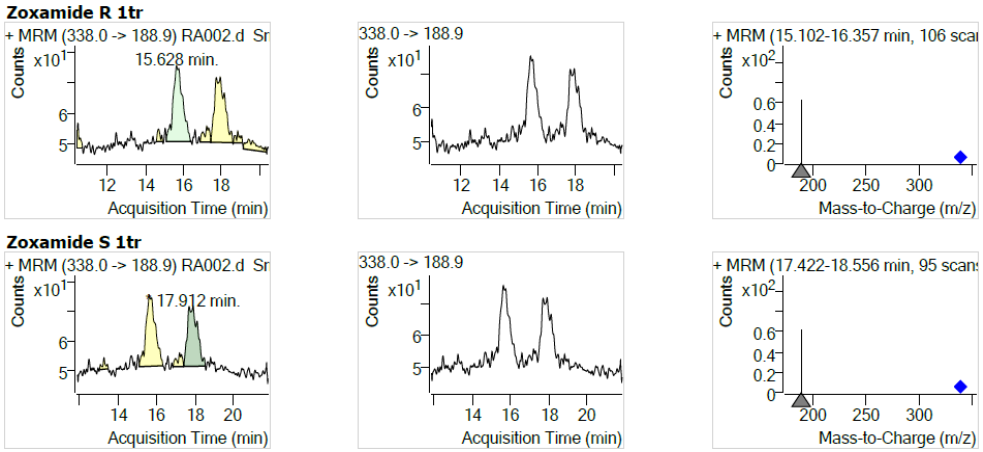


Figure A 7: Chromatogram of zoxamide (R/S)

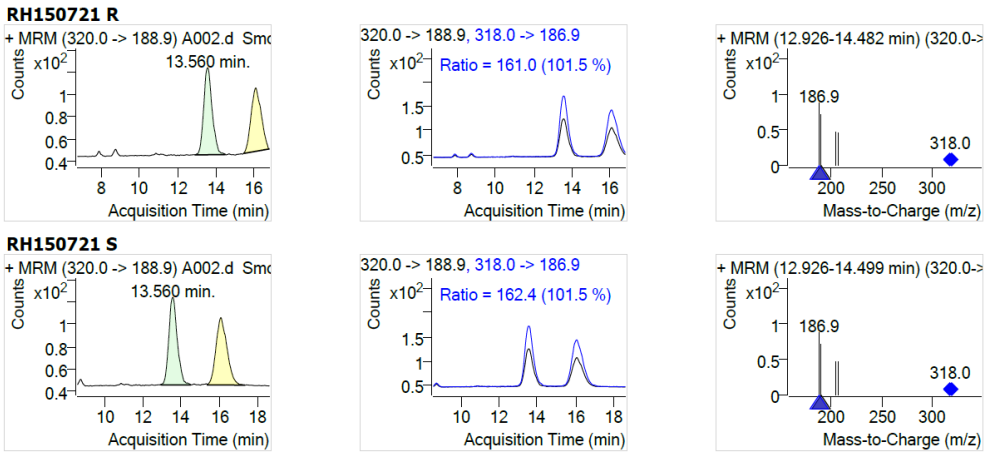


Figure A 8: Chromatogram of RH-150721 (R/S)

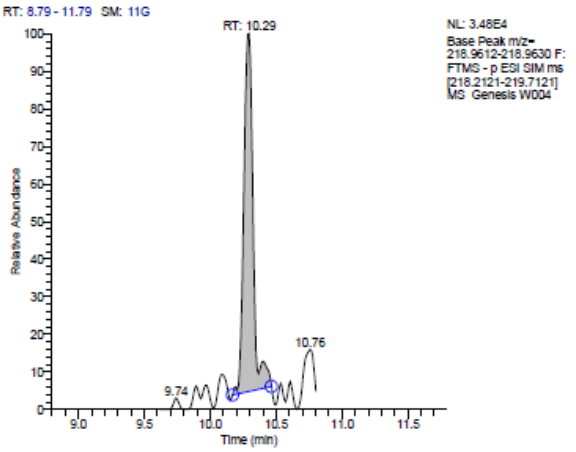


Figure A 9: Chromatogram of RH-141452

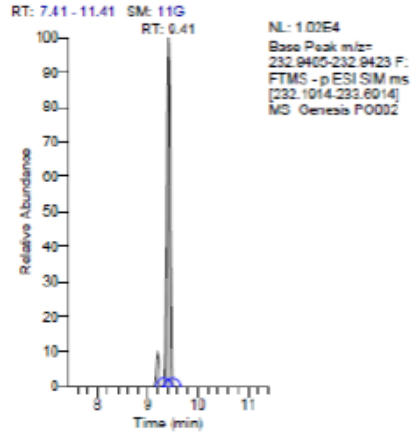


Figure A 10: Chromatogram of RH-141455

Conclusion

No residue of zoxamide and its metabolites RH-141452 and RH-141455 were found above LOD (0.003 mg/kg) in all the specimens (RAC specimens: potato tuber, processing specimens: potato flakes and French fries).

The method described was successfully validated according to the guidance SANCO/3029/99 rev. 4 (2000) and SANCO/825/00 rev. 8.1 (2010)

(Terranegra A. 2020)

(Pandolfi A. 2020)

(Sala A. 2021)

A 2.1.1.4 Description of analytical methods for the determination of residues in salad

A 2.1.1.4.1 Analytical method 1 - determination of zoxamide in salad (Agri BPL 040)

A 2.1.1.4.1.1 Method validation

~~Comments of zRMS:~~	~~The methods are acceptable.~~ ~~For evaluation of 5.1/26, 27 studies see Section 7 of the present RR.~~

Comments of zRMS:	The study is acceptable. No lettuces in the intended GAP and the presented 4 trials are from SEU, however they were used to generate substance specific DT50 values for fate modelling and worker exposure. Therefore, the study was evaluated. The method used was the validated in lettuce LC-MS/MS method. Two transitions were monitored (confirmatory method). The recoveries were within the required range.

Reference:	**KCP 5.1/26**
Report	Luciani, G.P., 2012: Determination of zoxamide and dimethomorph residues after two application of Zoxium 240 SC and GWN 9963 on lettuce, rocket salad and endive under field conditions – Italian trials, year 2012 Gowan Italia Spa, Italy AgriParadigma Srl, Italy, Report No AGRI 013/12 GLP DEC, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

And

Comments of zRMS:	The study is acceptable. No lettuces in the intended GAP and the presented 4 trials are from SEU, however they were used to generate substance specific DT50 values for fate modelling and worker exposure. Therefore, the study was evaluated. The method used was the validated in lettuce LC-MS/MS method. Two transitions were monitored (confirmatory method). The recoveries were within the required range.

Reference:	**KCP 5.1/27**
Report	Luciani G.P., 2012: Determination of zoxamide and dimethomorph residues after two application of Zoxium 240 SC and GWN 9963 on lettuce and rocket – Italian trials, year 2012 Gowan Italia Spa, Italy AgriParadigma Srl, Italy, Report No AGRI 014/12 GLP DEC, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010)

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical phase was conducted to determine the residues of zoxamide and dimethomorph in the field and greenhouse specimens on the basis of validated analytical method Agri BPL 040 Rev.6 in this study on lettuce and rocket salad under GLP compliance with guidelines SANCO/825/00 rev. 8.1 (2010).

Each specimen was taken out from the freezer, kept at room temperature for a few minutes. The specimen was taken and homogenized in a sample homogenizer.

Weigh 10.0 ± 0.l g for the sample previously homogenised into 50 ml Teflon centrifuge tube. Add 10 ml acetonitrile, shake sample vigorously for 1 min using Vortex mixer at maximum speed. Add 4 g anhydrous MgS04, lg NaCl, l g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate and mix on a Vortex mixer immediately for 5 min and centrifuge for 5minutes at > 3000 g.

Dimethomorph and zoxamide residue are quantified by high performance liquid chromatography coupled to a triple quadrupole mass spectrometer operating in Multiple Reaction Monitoring (MRM).

Limit of analytical quantification (LOQ) of zoxamide and dimethomorph in rocket salad and lettuce is 0.01 mg/kg.

Equipment

Instrument:	Waters ACQUITY UPLC System with XEVO TQ-S
Column:	Waters Acquity UPLC HSS T3. l.8 µm 2.1 x 100 mm
Mobile phase:	Solvent A: Methanol 10% & Water 90 % + 0.005 M ammonium forminate ($HCOONH_4$) Solvent B: Methanol + 0.005 M ammonium forminate (HCOONH4)

Time [min]	% A	% B
0.00	99.9	0.1
0.25	99.9	0.1
7.75	0.1	99.9
9	0.1	99.9
9.01	99.9	0.1
10.0	99.9	0.1

Flow rate:	450 µl/min
Column temp.:	40 °C
Injection volume:	10 µL
Retention time:	Dimethomorph: about. 6.1 – 6.3 min Zoxamide: about. 7.0 min
Ionisation:	ESI, Positive
Ion mode (SRM mode):	Zoxamide: m/z 336.1 → m/z 187.1 (quantifier ion) m/z 336.1 → m/z 159 (qualifier ion) Dimethomorph: m/z 388.1→ m/z 300.9 (quantifier ion) m/z 318588.1 → m/z (qualifier ion)

Results and discussions

Table A 28: Recovery results for zoxamide

Fortification level (F) mg/kg		Concentration	Residue found RF=C*V/W*d mg/kg	Mean value
Rocket salad				
A	0.0105	0.0041	0.008	**87.7**
	0.0105	0.0045	0.009	
	0.0105	0.0050	0.010	
	0.0105	0.0050	0.010	
	0.0105	0.0044	0.009	
B	41.6	0.0935	37.41	**92.1**
	41.6	0.0946	37.85	
	41.6	0.0980	39.19	
	41.6	0.1032	41.26	
	41.6	0.1034	41.35	
Lettuce				
A*	0.0052	0.0057	0.0057	**107.4**
	0.0052	0.0055	0.0055	
	0.0052	0.0055	0.0055	
	0.0052	0.0056	0.0056	
	0.0052	0.0056	0.0056	
B*	52	52.9	52.9	**94.4**
	52	50.5	50.5	
	52	48.8	48.8	
	52	47.8	47.8	
	52	48.2	48.2	

A: CV 9.1% A* CV: 1.5%
B: CV: 4.7 B* CV: 4.2%

Table A 29: Recovery results for dimethomorph

Fortification level (F) mg/kg		Concentration C	Residue found RF=C*V/W*d mg/kg	Mean value
Rocket salad				
A	0.0098	0.0048	0.0096	**101.0**
	0.0098	0.0049	0.0099	
	0.0098	0.0050	0.0100	
	0.0098	0.0050	0.0100	
	0.0098	0.0050	0.0100	

B	33.6	0.063	23.35	**78.9**
	33.6	0.065	26.05	
	33.6	0.065	26.20	
	33.6	0.068	27.26	
	33.6	0.069	27.65	
Lettuce				
A*	0.0056	0.0042	0.0042	**74.5**
	0.0056	0.0042	0.0042	
	0.0056	0.0043	0.0043	
	0.0056	0.0042	0.0042	
	0.0056	0.0041	0.0041	
B*	28	0.13	25.02	**83.7**
	28	0.12	23.69	
	28	0.11	22.88	
	28	0.11	22.64	
	28	0.11	22.98	

A: CV 1.8% A* CV: 1.7%
B: CV: 3.5 B* CV: 4.1%

Accuracy and precision / repeatability

	Precision	**Accuracy**
	CV%	**Recovery range**
Lettuce		
Zoxamide	4.2	70 – 110 %
Dimethomorph	4.1	70 – 110 %
Rocket salad		
Zoxamide	9.1	70 – 110 %
Dimethomorph	3.5	70 – 110 %

The lowest fortification level corresponds to the limit of quantification (LOQ).

Method precision is evaluated as coefficient of variation CV% or relative standard deviation RSD%. Recovery repeatability has been calculated analyzing 5 times the sample at the lowest fortification level.

All the recoveries were within the acceptable ranges of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was lower than 20%. The results obtained are in compliance with acceptability criteria reported in SANCO/825/00, rev 8.1.

Linearity

The response was found to be linear in the range of concentration 0.005 mg/L – 0.26 mg/L for zoxamide and from 0.006 mg/L – 0.28 mg/L for dimethomorph (6 concentration levels each). The correlation coefficient R^2 was well higher than 0.995.

Limit of quantification (LOQ)

Limit of analytical quantification (LOQ) of zoxamide and dimethomorph in rocket salad and lettuce is 0.01 mg/kg.

Matrix effects

Two independent analysis of blank sample per matrix were performed. No significant interferences exceeding 30% of the limit of quantification were determined in each sample at the retention time of benalaxyl-m and zoxamide in LC/MS analysis. However, matrix-matched standard solutions were used for analyte determination.

Specificity

The method can be regarded as specific with typical Rf values, typical mass spectra of the analytes and monitoring two ion transitions.

Stability of sample extracts and standard solutions

Final sample extracts were analysed within 24 hours.

Freezer storage of samples

The maximal storage interval between sampling and analysis was 54 days

Table A 30: Characteristics for the analytical method used for validation of zoxamide and dimethomorph residues in lettuce

	Zoxamide	Dimethomorph
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration. 6 point calibration, r^2 >0.995; linear Regression Model Individual calibration data and calibration line equation presented in the study report	Standard solution and matrix matched calibration. 6 point calibration, r^2 >0.995; linear Regression Model Individual calibration data and calibration line equation presented in the study report
Calibration range	0.005 mg/kg – 0.26 mg/kg	0.006 mg/kg – 0.28 mg/kg
Assessment of matrix effects is presented	Matrix matched standard solutions	Matrix matched standard solutions
Limit of determination/quantification	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg

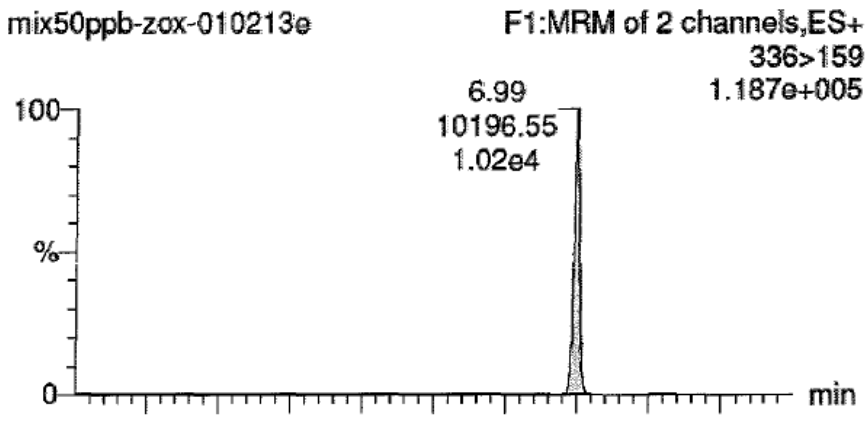


Figure A 11: **Chromatogram of zoxamide**

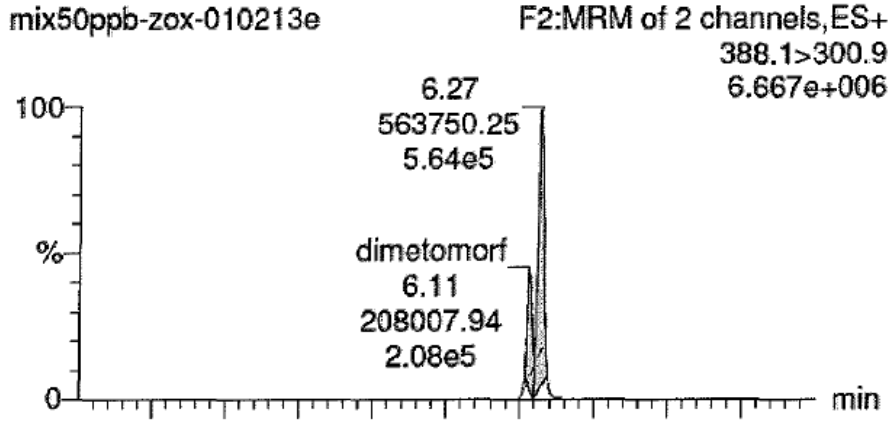


Figure A 12: **Chromatogram of dimethomorph**

Conclusion

The analytical method for the determination of zoxamide residues and its metabolites in lettuce (method Agri BPL 040 Rev.6) with LOW of 0.01 mg/kg has been sufficiently validated according to SANCO/825/00 rev. 8.1 (2010).

(Luciani G.P. 2012)

A 2.1.1.5 Description of analytical method for the determination of RH-129151 in plant extracts

In the following report the stability of RH-129151 in the final extract of grape, grape juice, wine, raisin, potato, potato flakes, tomato and cucumber samples has been studied. The analytical phase followed the method BPL-STUDY-18-000085. This method has been validated in line with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 for the determination of zoxamide and its metabolites (also (A)-RH-129151 and (B)-RH-129151) in a range of commodities. The method validation data are presented under KCP 5.2 (post-authorisation methods). The method principle is based on the QuEChERS-method using HPLC-MS/MS.

A 2.1.1.5.1 Analytical method 1

A 2.1.1.5.1.1 Method validation

~~Comments of zRMS:~~	~~The method is acceptable.~~ ~~For the evaluation please, see Section 7 of the current RR.~~

Comments of zRMS:	The study is acceptable. The method was validated in the same facility (BPL-STUDY-18-000085). No significative differences can be noticed between the results obtained at the end of the storage at 4°C (± 2°C) in the dark for at least 8 and 24 hours and the "time 0" values for all tested commodities. The % residue analyte after 8 and 24 hours is within the range of 70-120%, that is SANCO/825/00 rev. 8.1 guideline requirement to consider stable a chemical. Therefore, both the enantiomers of the analyte RH-

	129151 can be considered stable for 24 hours in the final extracts of the tested commodities and processed under the storage conditions of 4°C (± 2°C) in dark.

Reference:	**KCP 5.1/28**
Report:	Longhi, D., 2021: Evaluation of the stability of the analyte RH-129151 in the final extract of the following commodities and processed: grape, grape juice, wine, raisin, potato, fried potato, potato fries, tomato, peeled tomato, cucumber Gown Crop Protection Ltd., UK Lab Analysis s.r.l., Italy, Report no. GLP-STUDY-20-77, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010)
Deviations:	None.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The stability of the stability of the zoxamide metabolite RH-129151 (A, B and sum) in the final sample extract of grape (grape, grape juice, wine, raisin), potato (potato tuber, fried potato, potato flakes), tomato (tomato, peeled tomato), and cucumber commodities when stored for 8 and 24 hours under dark and refrigerated conditions at 4°C (± 2°C) has been studied by recovery experiments and analysed concurrently to freshly spiked samples. For the spiking, final extracts of each sample were divided into aliquots of 1 mL. Some aliquots were treated with about 30 µg/L of racemic RH-129151 (15 µg/L per enantiomer). Other control aliquots were kept untreated. Some samples were stored for 8 and 24 hours before analysis with method BPL-STUDY-18-000085, which has been validated according to SAN-CO/825/00 rev. 8.1 (2010).

The enantiomers of RH-129151, identified as RH-129151 (A) and RH-129151 (B) (since a clear identification of the order of elution of the enantiomers was not available at the moment of the emission of this document), were analysed according to the analytical method described in the LabAnalysis SOP P-AM-1323 and validated under GLP compliance according to SANCO/825/00 rev.8.1 and SANCO/3029/99 rev. 4. guidelines in the study BPL-STUDY-18-000085 “Validation of an analytical method to determine Zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities”.

The first step of the analytical method consisted in a double extraction of an aliquot of about 12.5 g of each matrix with about 15 mL of a mixture of Water/Acetonitrile/Methanol (20/40/40 v/v/v) containing 0.2% of Formic acid. The extracts were pooled and brought up to a final volume of 40 mL using the same solvent mixture. Only for potato flakes the amount weighed was of about 5 g, extracted twice with a volume of about 15 mL (first extraction) and 5 mL (second extraction), brought to a final volume of 20 mL. At each extraction, the separation of the liquid phase from the solid one was performed by centrifugation. On aliquots of the obtained extract an addition of both the analytes was carried out.

Following chromatographic method was used:

- Method B, for metabolite RH-129151, by chiral chromatography

The analytes were determined with instrumental methods B, detailed in the separate study summary of BPL-STUDY-18-000085.

Results and discussions

The obtained results of the spiking experiments are summarised in the following tables.

Table A 31: Recovery results of RH-129151 (A)

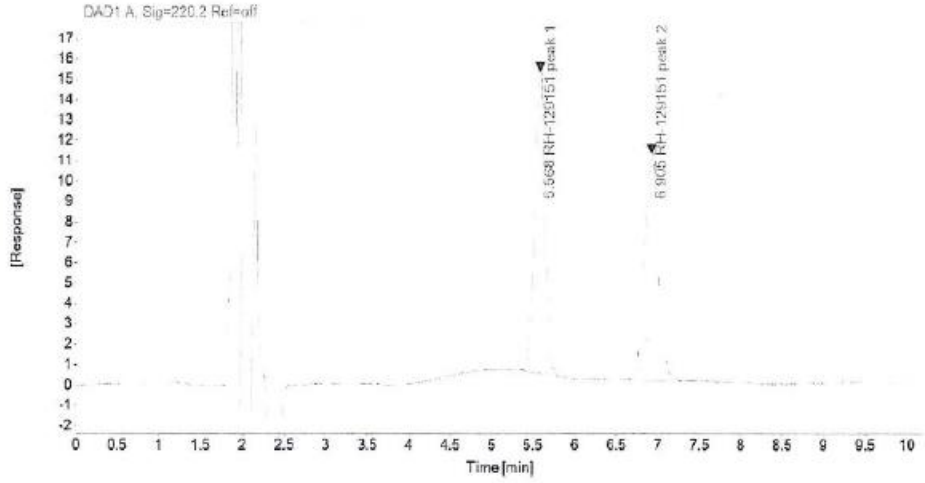
Commodity/processed	Commodity type	% Recovery analyte	
		T 8h	T 24 h
Grape	High acid content	102.1	101.1
Grape Juice	Processed	98.8	95.8
Wine	Processed	98.3	94.7
Raisin	Processed	98.6	97.1
Potato tuber	High water content	97.4	87.4
Fried potato	Processed	94.3	107.2
Potato flakes	Processed	106.9	95.4
Tomato	High water content	107.0	104.6
Peeled tomato	Processed	99.9	96.7
Cucumber	High water content	100.9	106.3

Table A 32: Recovery results of RH-129151 (B)

Commodity/processed	Commodity type	% Recovery analyte	
		T 8h	T 24 h
Grape	High acid content	96.4	93.8
Grape Juice	Processed	99.3	99.5
Wine	Processed	96.8	92.4
Raisin	Processed	100.2	101.0
Potato tuber	High water content	101.1	101.0
Fried potato	Processed	97.3	106.9
Potato flakes	Processed	99.7	93.8
Tomato	High water content	105.2	103.3
Peeled tomato	Processed	98.4	97.1
Cucumber	High water content	105.5	109.0

Please note: Since the enantiomers of RH-129151 could not be assigned as the R- and R-form (individual reference standards missing), they were assigned as “A” and “B”.

No significative differences in the recovery values were noticed after storage of the sample extracts at 4°C (± 2°C) in the dark for at least 8 and 24 hours compared to the initial “time 0” values for all the tested commodities: the % residue analyte after 8 and 24 hours is within the range of 70-120%, that is within the SANCO/825/00 rev. 8.1 guideline requirement to consider a chemical as stable. Therefore, RH-129151 (sum) as well as its enantiomer’s ratio was considered stable for 24 hours in the final sample extracts of the tested commodities when stored at 4°C (± 2°C) in dark.



Signal: DAD1 A, Sig=220,2 Ref=off

RT [min]	Area	Area %	Amount (mg/L)	Symmetry	Plates
5.568	131.14810	50.22	4.956	0.93	8764
6.905	130.00711	49.78	4.970	0.89	8004

Figure A 13: Chromatogram of chiral separation

Conclusion

The study demonstrates the stability of RH-129151 (sum) as well as its enantiomer's ratio in sample extracts of relevant crop commodities for 24 hours when stored at 4°C (± 2°C) in dark.

(Longhi D. 2021)

A 2.1.1.6 Description of analytical methods for the determination of residues in water, aqueous buffer solutions, aquatic media

A 2.1.1.6.1 Analytical method 1

A 2.1.1.6.1.1 Method validation

~~Comments of zRMS:~~	~~The method is acceptable.~~ ~~For the evaluation please, see Section 7 of the current RR.~~

Comments of zRMS:	The study is acceptable. The aim of this study was the determination of the effect of simulated processing conditions on the stability of the Zoxamide's metabolite RH-141452. The analytical method was based on HPLC-DAD system and it was validated according to the SANCO/825/00 rev.8.1. The validation parameters were within the required range. In the study no degradation products were found.

Reference:	**KCP 5.1/29**
Report	Longhi, D., 2019: RH-141452: Hydrolysis under simulated processing conditions Gowan Crop Protection Ltd, UK

	LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-18-000092, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Aqueous buffer samples containing RH-141452 were directly injected in a HPLC-DAD system. Concentrations of the test item were determined using matrix-matched calibration solutions. The analytical method with an LOQ of 1 mg/L in all buffers (pH 4, pH 5 and pH 6) was validated according to the SANCO/825/00 rev. 8.1 guideline. The identity of the analyte was confirmed by a high-resolution mass spectrometer detector and a DAD detector recording the UV spectra between 200 and 400 nm. Both the MS and the UV spectra of the reference standard and of the test samples were compared. The same profile confirmed the identity of the analyte in the test item sample.

Equipment

Instrument:	Agilent HPLC 1260 Infinity II
Column:	Poroshell 120 EC-C18, 4 µm, 4.6 x 100 mm;
Column temp.:	25°C
Mobile phase:	A: 0.1 % v/v phosphoric acid in UPLC grade water (prepared dissolving a volume of 2.5 mL of phosphoric acid in 2.5 L of water); B: 0.1 % v/v phosphoric acid in HPLC grade acetonitrile (prepared dissolving a volume of 2.5 mL of phosphoric acid in 2.5 L of acetonitrile);

Elution gradient:

Time (min)	% A	% B
0	98	2
4	98	2
10	0	100
13	0	100

Flow rate:	1 mL/min
Run time:	3 minutes
Injection volume:	20 µL
Detector type:	Diode array detector (DAD)
Retention time:	RH-141452 ~8.3 minutes

Results and discussions

Table A 33: Summary of recovery experiments

Matrix	Fortification Level (mg/L)	Accuracy and precision per level		Overall accuracy and precision	
		Accuracy (%) n=5	RSD (%) n=5	Mean Accuracy (%) n=10	RSD (%) n=10
Buffer pH 4	1 (LOQ)	96.89	3.26	98.5	2.8
	10 (10 x LOQ)	100.2	0.4		
Buffer pH 5	1 (LOQ)	98.83	0.69	99.6	1.0

	10 (10 x LOQ)	100.3	0.6		
Buffer pH 6	1 (LOQ)	96.95	3.43	98.9	3.0
	10 (10 x LOQ)	100.8	0.4		

Accuracy and precision / repeatability

The results for accuracy and precision showed RSD values of <10% for each spiking level and each buffer and recovery values within the required range of 70-110%.
Two untreated samples of each buffer were analysed, resulting in values < LOD.

Linearity

Linearity was checked by a 6-points calibration curve (single injections) using matrix matched standard solutions at a range of 0.3024 – 15.12 mg/L for each buffer ($R^2 > 0.99$). They had a range from 30 % of the LOQ to > 20 % above the highest level (i.e. 10 mg/L).

Limit of quantification

The limit of quantification of this method is 1 mg/L of RH-141452 in all the buffers.

Limit of detection

The limit of detection was tested in matrix matched standard solutions (for each buffer) at a concentration of 0.1008 mg/L. It corresponds to 10% of the LOQ and is not higher than 30% of the LOQ value.

Matrix effects

Analytes were measured by direct injection of the (matrix) samples and matrix-matched external standard solutions were used.

Specificity

Interference was checked by comparing the chromatograms of matrix-matched analyte solutions at LOQ level to chromatograms obtained from blank samples for each buffer. As a result, no interfering signals in amounts higher than 30% of the LOQ level were detected in the untreated matrix.

The identity of the analyte was confirmed by a high-resolution mass spectrometer detector and a DAD detector recording the UV spectra between 200 and 400 nm. Both the MS and the UV spectra of the reference standard and of the test samples were compared. The same profile confirmed the identity of the analyte in the test item samples. Typical spectra are shown below.

Storage of samples and storage stability

Not applicable. Aqueous buffer samples were directly after sampling injected in a HPLC-DAD system.

Table A 34: Characteristics of the analytical method validation for the determination of RH-141452 in aqueous buffer samples

	RH-141452
Specificity	Mass spectrum provided. Blank value < 30% LOQ.
Calibration (type, number of data points)	Matrix matched standard calibration. 6 point calibration; r^2 >0.99; linear. Calibration data and calibration line equation presented in the study report.
Calibration range	0.3024 – 15.12 mg/L
Assessment of matrix effects is presented	No. (Matrix matched standard clibration.)

	RH-141452
Specificity	Mass spectrum provided. Blank value < 30% LOQ.
Limit of determination/quantification	LOQ: 1 mg/L LOD: 0.1008 mg/L

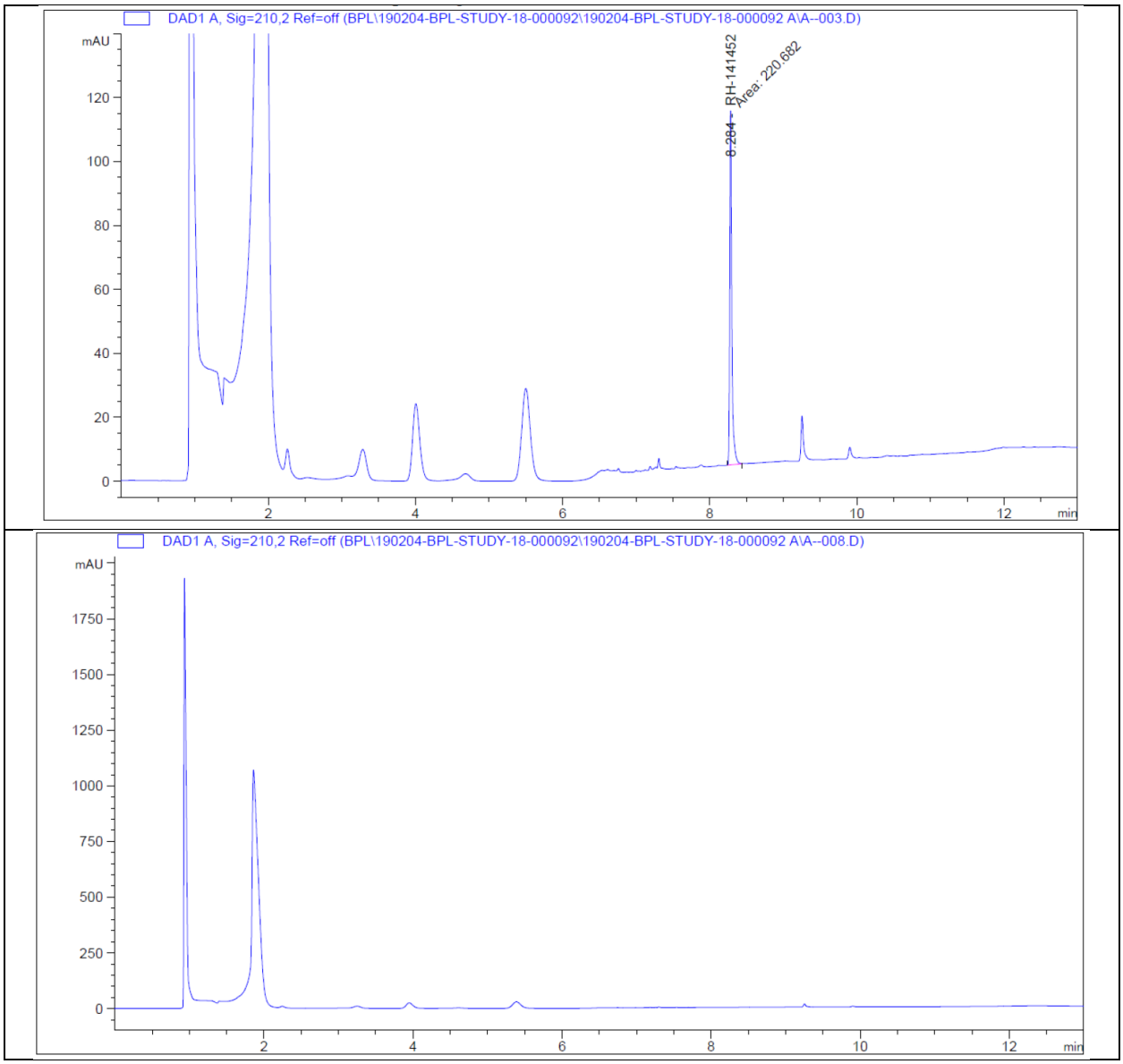


Figure A 14: Representative UV DAD chromatograms showing RH-141452 standard at 1 mg/L (LOQ) in buffer solution of pH 4 (above) and matrix blank (below)

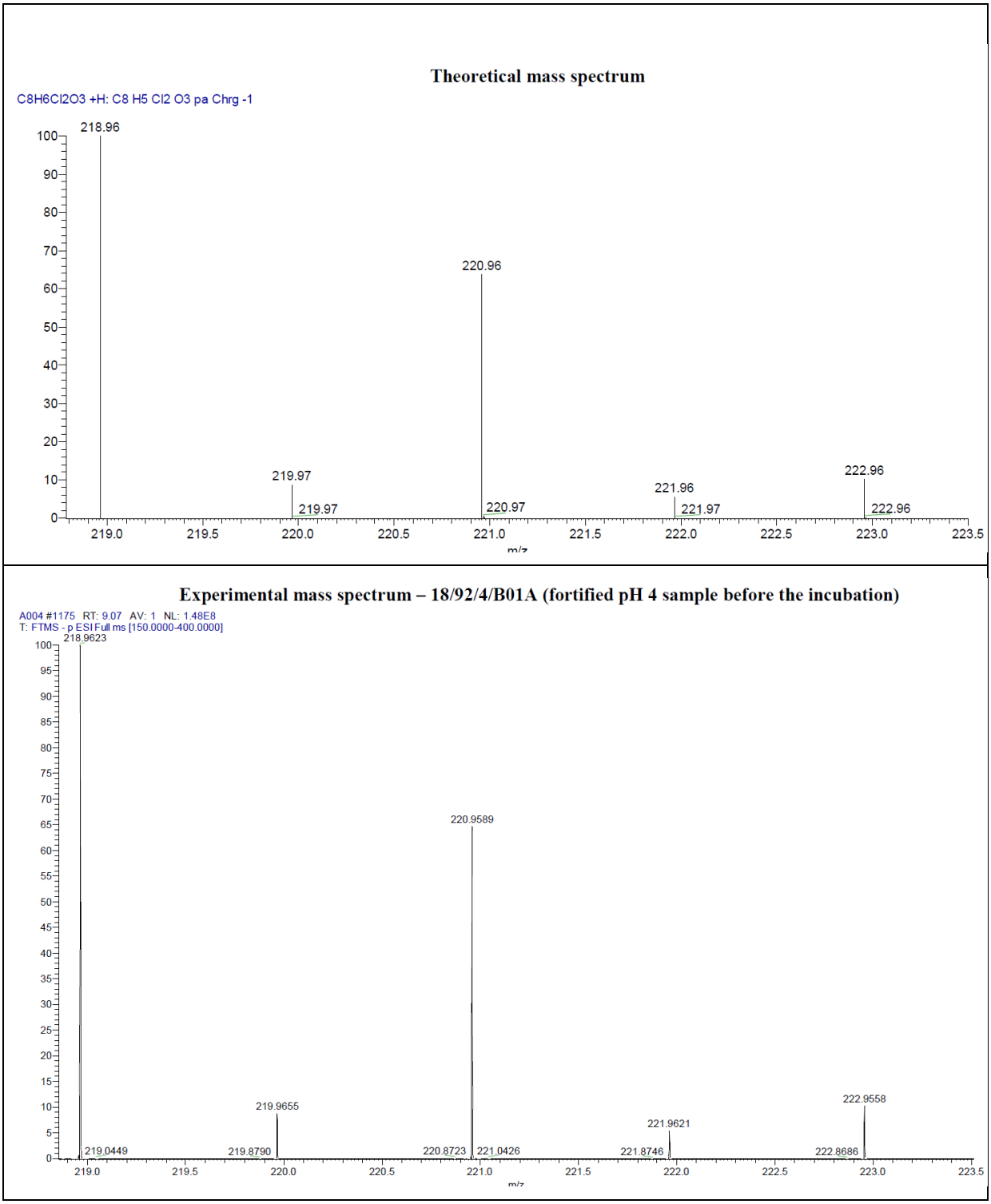


Figure A 15: Theoretical MS spectrum of RH-141452 (above) and spectrum of a RH-141452 in a fortified buffer solution at pH 4 (below)

Conclusion

The analytical method has been sufficiently validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) for the monitoring of RH-141452 in aqueous buffer solutions at different pH.

(Longhi D. 2019)

A 2.1.1.6.2 Analytical method 2

A 2.1.1.6.2.1 Method validation

~~Comments of zRMS:~~	~~The method is acceptable.~~ ~~For the evaluation please, see Section 7 of the current RR.~~

Comments of zRMS:	The study is acceptable. The aim of this study was the determination of the effect of simulated processing conditions on the stability of the Zoxamide's metabolite RH-141455. The analytical method was based on HPLC-DAD system and it was validated according to the SANCO/825/00 rev.8.1. The validation parameters were within the required range. In the study no degradation products were found.

Reference:	**KCP 5.1/30**
Report	Longhi, D., 2019: RH-141455: Hydrolysis under simulated processing conditions Gowan Crop Protection Ltd, UK LabAnalysis s.r.l., Italy, Report No. BPL-STUDY-19-000009, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Aqueous buffer samples containing RH-141455 were directly injected in a HPLC-DAD system. Concentrations of the test item were determined using external calibration solutions. The analytical method with an LOQ of 1 mg/L in all buffers (pH 4, 5, 6) was validated according to the SANCO/825/00 rev. 8.1 guideline. The confirmation of the analyte identification was performed using a high-resolution mass spectrometer detector and a DAD detector recording the UV spectra between 200 and 400 nm. Both the MS and The UV spectra of the reference standard and of the test sample were compared. The same profile confirmed the identity of the analyte in the test item sample.

Equipment

Instrument:	Agilent HPLC 1260 Infinity II
Column:	Poroshell 120 EC-C18, 4 µm, 4.6 x 100 mm;
Column temp.:	25°C
Mobile phase:	A: 0.5 % v/v phosphoric acid in UPLC grade water (prepared dissolving a volume of 12.5 mL of phosphoric acid in 2.5 L of water); B: 0.1 % v/v phosphoric acid in HPLC grade acetonitrile (prepared dissolving a volume of 2.5 mL of phosphoric acid in 2.5 L of acetonitrile);

Gradient:

Time (min)	% A	% B
0	98	2
5	98	2

12	0	100
14	0	100

Flow rate: 1 mL/min
Post time: 3 minutes
Injection volume: 20 µL
Retention time: 9.1 minutes
Detector type: Diode array detector (DAD)

Results and discussions

Table A 35: Summary of recovery experiments

Matrix	Fortification Level (mg/L)	Accuracy and precision per level		Overall accuracy and precision	
		Accuracy (%) n=5	RSD (%) n=5	Mean accuracy (%) n=10	RSD (%) n=10
Buffer pH 4	1 (LOQ)	99.13	3.62	99.29	2.46
	10 (10 x LOQ)	99.45	0.70		
Buffer pH 5	1 (LOQ)	100.5	1.5	98.60	2.8
	10 (10 x LOQ)	96.63	2.54		
Buffer pH 6	1 (LOQ)	91.08	0.39	94.99	4.73

Accuracy and precision / repeatability

The results for accuracy and precision showed RSD values of <10% for each spiking level and each buffer and recovery values within the required range of 70-110%.
Two untreated samples for each buffer were analysed, resulting in values < LOD.

Linearity

Linearity was checked by a 6-point calibration curve (single injections) over the range 0.3036 – 15.18 mg/L for each buffer (R^2 > 0.99). They had a range from 30 % of the LOQ to > 20% above the highest residue level (i.e. 10 mg/L).

Limit of quantification

The limit of quantification of this method is 1 mg/L for RH-141455 in all the buffers.

Limit of detection

The limit of detection was tested for each buffer at a concentration of 0.1012 mg/L. It corresponds to 10 % of the LOQ and is not higher than 30 % of the LOQ value.

Matrix effects

Matrix effects were not significant (< 20%), therefore calibration with standards in solvent were used.

Specificity

Interference was checked by comparing the chromatograms of analyte solutions at LOQ level to chromatograms obtained from blank samples for each buffer. As a result, no interfering signals in amounts higher than 30% of the LOQ level were detected in the untreated matrix.

The identity of the analyte was confirmed by a high-resolution mass spectrometer detector and a DAD detector recording the UV spectra between 200 and 400 nm. Both the MS and the UV spectra of the reference standard and of the test samples were compared. The same profile confirmed the identity of the analyte in the test item samples. Typical spectra are shown below.

Storage of samples and storage stability

Not applicable. Aqueous buffer samples were directly after sampling injected in a HPLC-DAD system.

Table A 36: Characteristics of the analytical method validation for the determination of RH-141455 in aqueous buffer samples

	RH-141455
Specificity	Mass sprectrum provided. Blank values < 30% LOQ.
Calibration (type, number of data points)	Standard solution calibration. 6 point calibration range; r^2 >0.99; linear Calibration data and calibration line equation presented in the study report.
Calibration range	0.3036 – 15.18 mg/L
Assessment of matrix effects is presented	Yes. (Matrix effects were not significant (< 20%).)
Limit of determination/quantification	LOQ: 1 mg/L LOD: 0.1012 mg/L

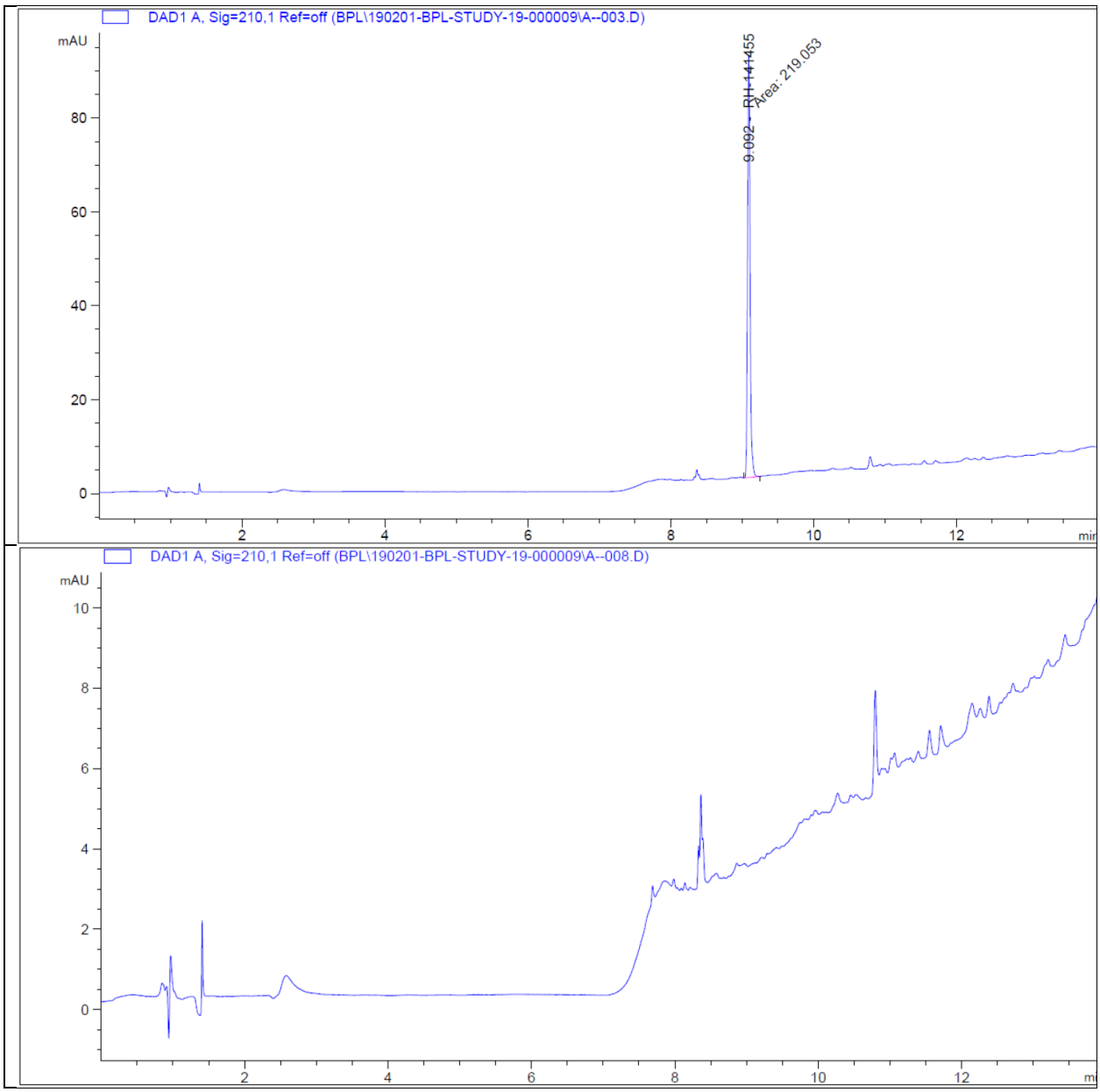


Figure A 16: Representative UV DAD chromatograms showing RH-141455 standard at 1 mg/L (LOQ) in solution (above) and solvent blank (below)

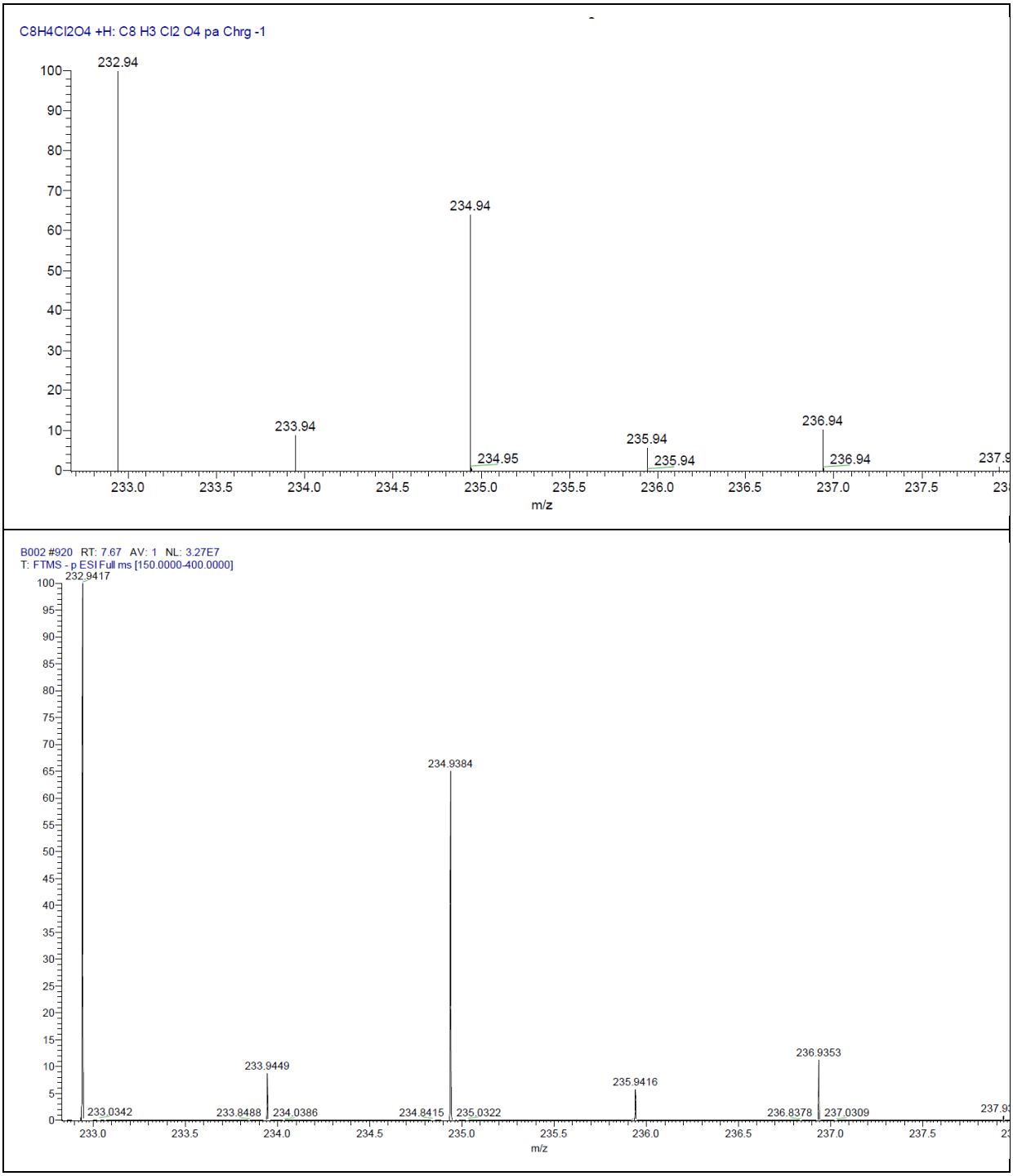


Figure A 17: Theoretical MS spectrum of RH-141455 (above) and spectrum of a RH-141455 in standard solution (acetonitrile) (below)

Conclusion

The analytical method has been sufficiently validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) for the monitoring of RH-141455 in aqueous buffer solutions at different pH.

(Longhi D. 2019)

A 2.1.1.6.3 Analytical method 3

A 2.1.1.6.3.1 Method validation

Comments of zRMS:	The study is zoxamide confirmatory study currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/31**
Report	Cashmore, A., 2019: RH-141288: partition coefficient (n-octanol/water): Shake Flask Method Gowan Crop Protection Ltd, UK Smithers ERS Ltd., UK, Report No. 3202371, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical procedure (SMV (PC) 3202371-01V) for the determination of Zoxamide metabolite RH-141288 in pH 5, 7 and 9 buffers saturated with n-octanol and n-octanol saturated with pH 7 buffer, was established (under Smithers ERS non-GLP study 3202370) and validated in this study with regards to accuracy, precision, linearity, specificity and limit of quantification (LOQ).

Control samples of pH 5, 7 and 9 buffers saturated with n-octanol and n-octanol saturated with pH 7 buffer were fortified with RH-141288 at three different concentrations in quintuplicate and analysed. Samples were diluted into calibration range with acetonitrile: water (75:25 v/v). Analysis was by High Precision Liquid Chromatography with UV detection (HPLC-UV).

Mean recoveries were within the acceptable range of 70-110% at each concentration and for each test matrix. Recoveries met the ideal criteria of ≥ 90% for pH 5 buffer, pH 9 buffer and n-octanol. Recovery did not meet the ideal criteria for pH 7 buffer, but was still within the acceptable range of 70-110%.

Equipment

Instrument:	Agilent series 1100 HPLC system
Column:	X-Bridge shield RP18, 3.5 µm, 150 mm × 4.6 mm
Mobile phase:	A: Double distilled water (25%) B: Acetonitrile (75%)
Flow rate:	1.0 mL/min
Column temp.:	25°C
Injection volume:	25 µL
Retention time:	approx. 2.5 min
Analysis time:	5 min
UV wavelength:	240 nm

Results and discussions

Table A 37: Summary of validation results

Test Matrix	Validation level						
	Low		Middle		High		
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	r^2
pH 5 buffer	98.46	5.00	105.2	3.15	104.7	3.82	0.9998
pH 7 buffer	88.92	4.26	101.0	2.01	99.88	3.35	
pH 9 buffer	95.33	6.86	104.1	1.50	102.3	3.08	
n-Octanol	102.8	0.613	101.4	1.26	101.6	1.28	

For buffer saturated with n-octanol the low, middle and high validation levels were 1, 5 and 10 µg/mL respectively. For n-octanol saturated with pH 7 buffer, the low, middle and high validation levels were ca. 2000, 3000 and 4000 µg/mL respectively.

Accuracy and precision / repeatability

Mean recoveries were within the acceptable range of 70-110% at each concentration and for each test matrix. Recoveries met the ideal criteria of ≥ 90% for pH 5 buffer, pH 9 buffer and n-octanol. Recovery did not meet the ideal criteria for pH 7 buffer, but was still within the acceptable range of 70-110% stated in the study protocol.

Precision met the acceptance criteria of ≤ 20% (and met the ideal criteria of ≤ 10%) at each concentration and for each test matrix.

The SANCO 3029/99 guideline allows one significant outlier to be discarded from each validation level. One significant outlier was removed from the pH 7 buffer top and middle levels, and one from the pH 9 low level. It was suspected that the affected samples had been incorrectly diluted, however as there was sufficient data to meet the validation criteria, the samples were not re-analysed.

Linearity

The response of the HPLC-UV system to RH-141288 was linear over the range of 0.1 to 10 µg/mL using a 1/x weighting. The coefficients of determination (r^2) were ≥ 0.98.

Limit of quantification

The LOQ was determined to be 1 µg/mL for pH 5, 7 and 9 buffers saturated with n-octanol and 2000 µg/mL for n-octanol saturated with buffer. The LOQ is the lowest sample concentration at which precision, accuracy and specificity criteria were demonstrated to be acceptable.

Matrix effects

Matrix-matched standards were used.

Specificity

No significant interferences at the retention time of RH-141288 were found in blank sample matrices at > 30% of the LOQ peak area response. The method was therefore considered to be specific for RH-141288.

Table A 38: Characteristics of the analytical method validations for RH-141288 in n-octanol/water

	RH-141288
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 9-point calcibrations, linear with 1/x weighting; r > 0.98. Calibration data and calibration line equations presented in the study reports.
Calibration range	0.1 – 10 µg/mL
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ)	1 µg/mL (buffers saturated with n-octanol) 2000 µg/mL (n-octanol saturated with buffer)

The following figures show typical chromatograms.

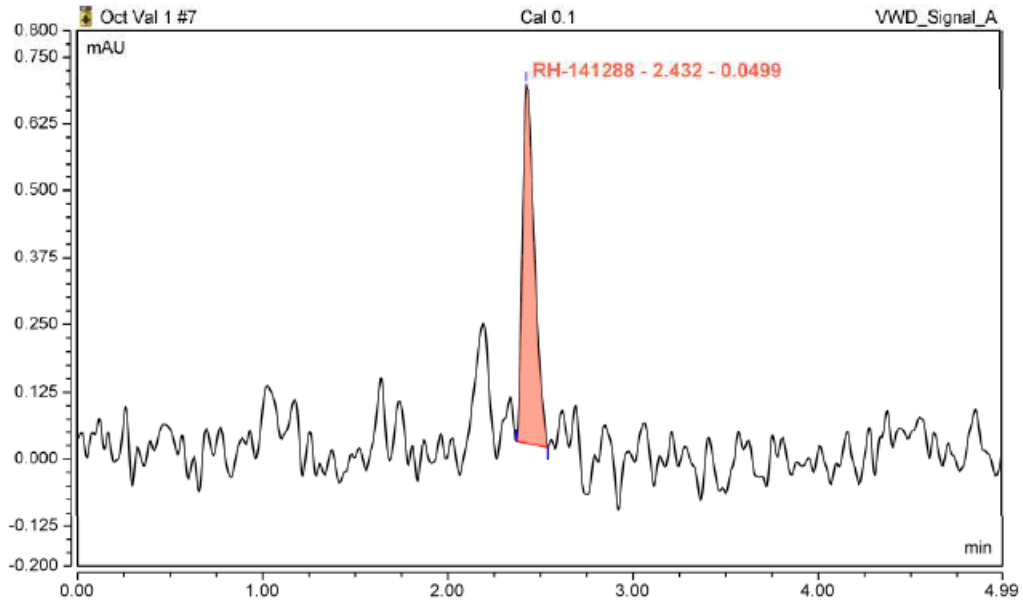


Figure A 18: **Typical calibration standard for RH-141288 at 0.1 µg/mL**

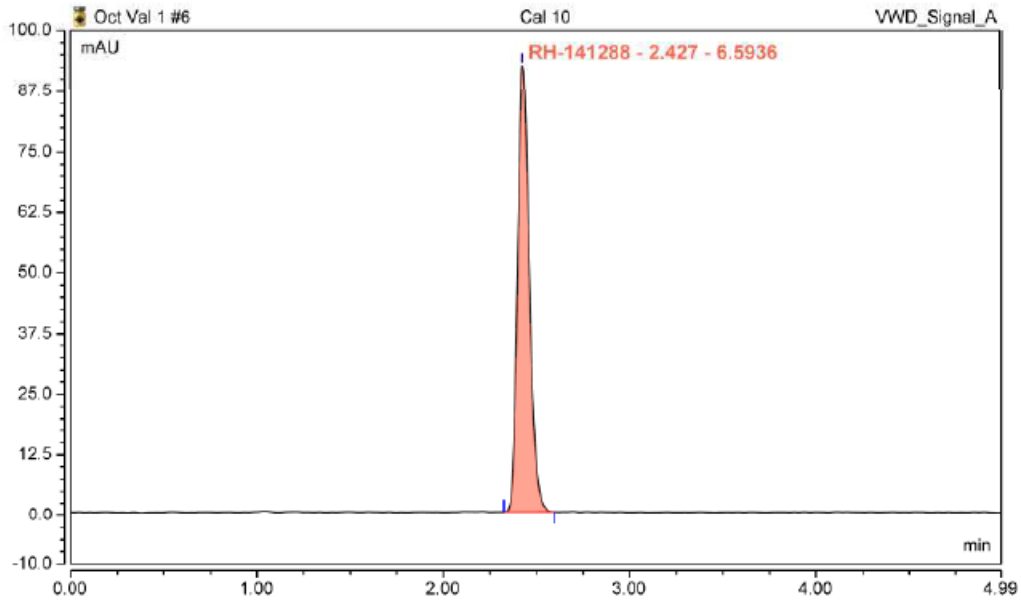


Figure A 19: **Typical calibration standard for RH-141288 at 10 µg/mL**

Conclusion

The analytical procedures for the determination of RH-141288 in pH 5, 7 and 9 buffers saturated with n-octanol and n-octanol saturated with pH 7 buffer have been successfully validated according to SANCO 3029/99 rev 4.

(Cashmore A. 2019)

A 2.1.1.6.4 Analytical method 4

The following method has been validated and used for the determination of RH-163353 in aquatic media.

It has also been provided to Latvia as RMS for zoxamide for interzonal evaluation in July 2020.

A 2.1.1.6.4.1 Method validation

Comments of zRMS:	The studies 5.1/32, 33, 34, 35 are zoxamide confirmatory studies currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/32**
Report	xxx., 2020: RH-163353: Fish acute toxicity test Gowan Crop Protection Ltd., UK xxx, Report No. 3202385, GLP, Not published
Guideline(s):	SANCO 3029/99 rev 4 (2000)
Deviations:	No
GLP	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/33**
Report	Jarrom, R., 2020: RH-163353 Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202386, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	A temperature deviation was noted during the range finding and definitive test because the continuous temperature vessel variation was >2°C (range finding test = 2.3°C; definitive test = 2.1°C). This slight deviation (protocol specification = within 2°C) was not considered to have had an impact on the integrity of the study.
GLP	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/34**
Report	Jarrom, R., 2020: RH-163353: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202387, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4 (2000)
Deviations:	The measured pH of the 100 mg/L solution at test initiation was 7.37 (0.13 outside of the specified range of 7.5 – 8.5), pH adjustment was not made. This protocol deviation has no impact on the integrity of the study as no mortality was seen in the 100 mg/L replicates, demonstrating no negative effects on the test organisms.
GLP	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/35**
Report	Jarrom, R., 2020: RH-163353: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202388, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	Additional light measurements were taken at 24 and 48-hours to ensure the light remained within the correct ranges. The maximum temperature recorded during the test was 24.6°C and therefore, this exceeded the protocol range (21–24°C). This temperature deviation occurred within the first 45-hour of the test. As the test temperatures were measured at approximately 24-hour intervals it is not possible to confirm the exact length of the out of specification period, however, the temperature was out of specification (ac-

	tual temperature = 24.4°C) when recorded at ca 21-hours but was back in specification (actual temperature = 23.4°C) when recorded at 45-hours. As the temperature remained within 2°C, the algae growth was good and achieved all the control validity criteria, this protocol deviation was not considered to have an impact on the integrity of the study.
GLP	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202385-01V, SMV 3202386-01V, SMV 3202387-01V and SMV 3202388-01V and following versions were used to determine the concentration of the analyte RH-163353 in samples of test media from fish acute, *Daphnia* acute, mysid and alga growth studies, respectively. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-163353 were determined by treating samples with acetonitrile containing 0.5% formic acid, then diluting further with acetonitrile/aquatic medium (1:4 v/v) containing 0.1% formic acid as required to bring the response within the calibration range. Samples were analysed by liquid chromatography-time of flight mass spectrometry (LC-TOF/MS).

RH-163353 is a racemate. The analytical method validation for the enantiomeric ratio analysis of RH-163353 was conducted under Smithers ERS Study Number 3202586 (established analytical procedure, SMV 3202586-01V and following) by inclusion of an enantioselective column. A combination of the above-mentioned analytical procedures with SMV 3202586 was used to assess in addition the enantiomeric ratio of the test substance in the test media and calibration standard solution.

Equipment (LC-TOF/MS Analysis)

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Waters BEH Phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase:	A: 0.1% Formic acid in water (LC-MS grade) B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	20 µL (fish, mysid, alga), 15 (*Daphnia*)
Retention time:	approx. 2.16 min
Analysis time:	5 min
Ionisation:	Electrospray (ESI), positive
Ion mode	Product of 332.04 Da: 186.939 – 186.989 Da

For further MS conditions, please refer to the study report.

Equipment for enantiomeric ratio analysis

Instrument:	AB Sciex TripleTOF 5600+ coupled to Shimadzu SIL-30ACMP Quaternary HPLC
Column:	Phenomenex Lux i-Cellulose 5, 150 x 4.6 mm, 5 µm
Mobile phase:	A: 0.1% Formic acid in water (LC-MS grade) B: 0.1% Formic acid in acetonitrile (LC-MS grade) Isocratic elution: %A 60; %B 40
Flow rate:	1.0 µL/min
Column temp.:	25°C
Injection volume:	40 µL
Retention time:	Isomer A: 7.42 min Isomer B: 8.08 min
Analysis time:	15 min
Ionisation:	Electrospray (ESI), positive
Ion mode:	Product of 332.04 Da: 186.939 – 186.989 Da

Results and discussions

Table A 39: Summary of recovery experiments in the fish study

Treated mains water	0.001 µg/mL	1.0 µg/mL	150 µgm/L	Overall
Mean Recovery (%)	100	103	103	102
CV (%)	7.07	4.30	4.12	5.13

Table A 40: Summary of recovery experiments in the daphnid study

Elendt medium	0.1 µg/mL	1.0 µg/mL	150 µg/mL	Overall
Mean Recovery (%)	96.9	99.3	88.5	94.9
CV (%)	3.09	8.65	4.77	7.59

Table A 41: Summary of recovery experiments in the mysid study

Brackish water	0.1 µg/mL	5.0 µg/mL	150 µg/mL	Overall
Mean Recovery (%)	102	101	106	103
CV (%)	2.03	1.81	1.43	2.66

Table A 42: Summary of recovery experiments in the alga study

EC medium	0.1 µg/mL	5.0 µg/mL	150 µg/mL	Overall
Mean Recovery (%)	102	99.0	97.6	99.5
CV (%)	2.10	1.54	1.81	2.55

Accuracy and precision / repeatability

The accuracy and repeatability / precision of the procedure were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response of the LC-TOF/MS analysis was determined over the concentration ranges of 0.0001 – 0.1 µg/mL for alga, fish and mysid, and over the concentration range 0.001 – 0.5 µg/mL for *Daphnia* for the respective media solutions.

At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting.

All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ was set to 0.001 mg/L for fish and 0.1 mg/L for *Daphnia*, mysid and alga.

Matrix effects

Matrix-matched standards were used.

Specificity

The LC-TOF/MS analysis method was considered specific as the analysis of treated mains water exhibited no response exceeding 30% of the proposed LOQ of 0.0001 µg/mL for fish and of 0.1 µg/mL for daphnia, mysid and alga from either the test substance or interferences at the retention times of each component.

Storage stability

Sample extract stability of RH-163353 at around +4°C was demonstrated over a period of 7 days for fish and mysids samples, 9 days for alga samples and 12 days for *Daphnia* samples. Samples were stored in glass vials.

Frozen storage stability of samples at ≤-10°C was demonstrated over a period of 28 days for fish samples, and over 31 days for *Daphnia*, mysid and alga samples.

Table A 43: Characteristics of the analytical method validations for RH-163353 in aquatic media

	RH-163353
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-point calcibrations, linear with 1/x weighting; r > 0.99. Calibration data and calibration line equations presented in the study reports.
Calibration range	0.0001 – 0.1 µg/mL (fish, mysid, alga) 0.001 – 0.5 µg/mL (*Daphnia*)
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ)	0.001 mg/L (fish) 0.1 mg/L (*Daphnia*, mysid, alga)

The following figures show typical chromatograms.

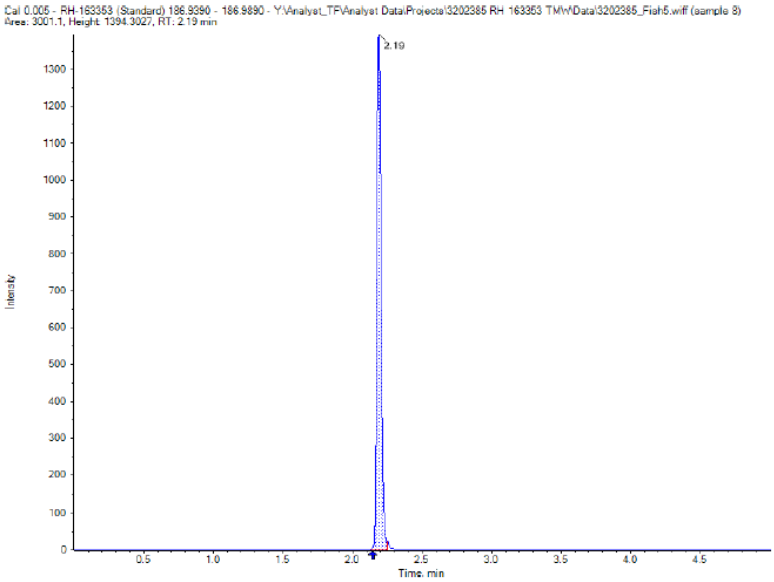


Figure A 20: **Chromatogram of RH-163353 (sum of isomers)**

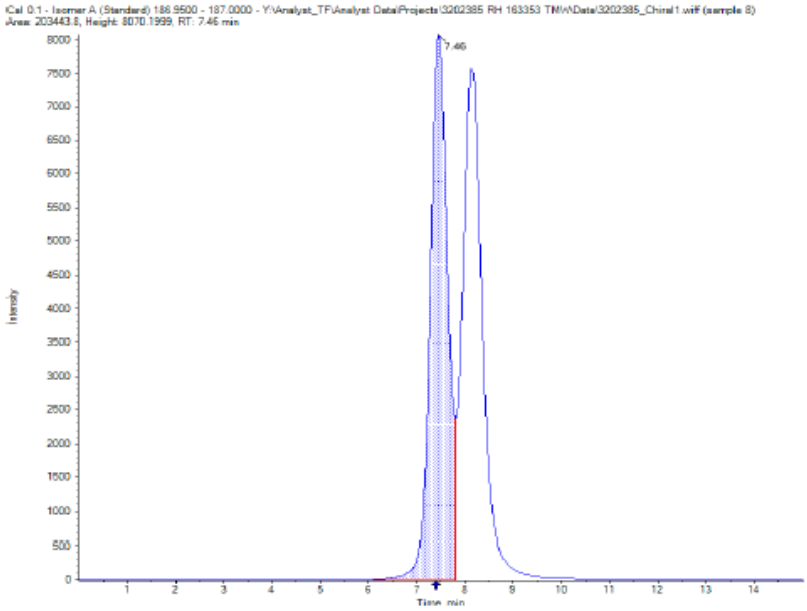


Figure A 21: **Chromatogram of Isomer A**

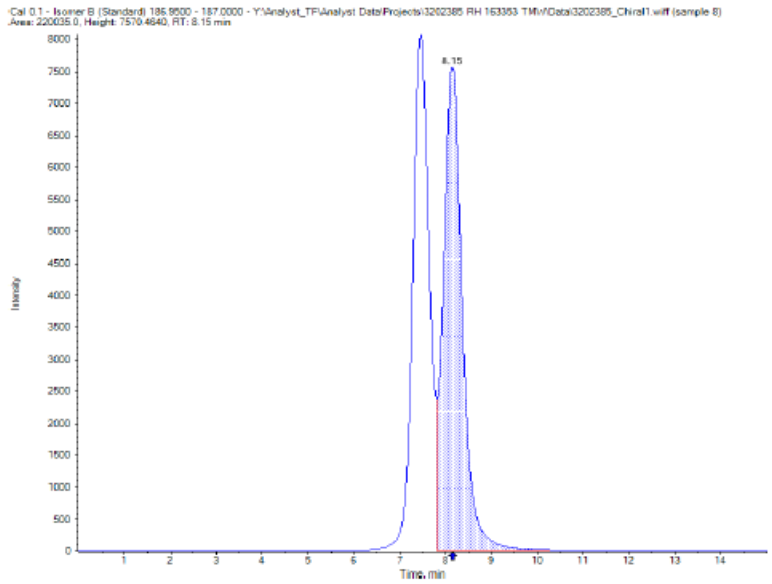


Figure A 22: **Chromatogram of Isomer B**

The enantiomeric ratio analysis confirmed the stability of the chiral C of RH-163353 – it stayed in the test media as racemate over the test periods. Typical results (examples) of the measurements are summarised in the following tables.

Table A 44: Enantiomeric ratio of 96-hour sample in fish medium

Nominal Concentration (mg/L)	% of Total	
	Isomer A	Isomer B
0.001 (calibration standard)	49.81	50.19
0.5 (calibration standard)	48.04	51.96
Mean	48.93	51.08
+3SD*	50.43	52.58
-3SD*	47.4	49.58
100 (test sample)	48.1	51.9

* SD = Standard deviation. Taken from enantiomeric ratio method (Study 3202586), where the value determined was 0.5 (also see GLP Certificate of Analysis)

Table A 45: Enantiomeric ratio of 48-hour samples in *Daphnia* medium

Nominal Concentration (mg/L)	% of Total	
	Isomer A	Isomer B
100	48.1	51.9

Conclusion

The analytical procedures for the determination of RH-163353 in aquatic media have been successfully validated according to SANCO 3029/99 rev 4.

(xxx. 2020, xxx 2020a,b,c)

A 2.1.1.6.5 Analytical method 5

The following method has been validated and used for the determination of RH-141455 in aquatic me-dia.

It has also been provided to Latvia as RMS for zoxamide for interzonal evaluation in July 2020.

A 2.1.1.6.5.1 Method validation

Comments of zRMS:	The studies 5.1/36, 37, 38 are zoxamide confirmatory studies currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/36**
Report	xxx, 2020: RH-141455: Fish acute toxicity test Gowan Crop Protection Ltd., UK xxx, UK, Report No. 3202716, GLP, Not published
Guideline(s):	SANCO 3029/99 rev 4 (2000)
Deviations:	No
GLP	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/37**
Report	Hugill, E., 2019: RH-141455: Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202380, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	There was no confirmation in the range finding data that the vitamin stock was added to the Elendt M4 media by error. This protocol deviation has no impact on the integrity of the study because the main test was performed without deviation and the immobilisation at all treatments was ≤ 10% - indicating that there was no impact on *Daphnia* if the vitamin stock was not added. A temperature deviation was noted during the test because the 100 mg/L treatment vessel temperature deviated by 2.2°C from the initial vessel temperature. This slight deviation (protocol specification = within 2°C) was not considered to have had an impact on the integrity of the study, as there was not test substance immobilisation during the test.
GLP	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/38**
Report	Hugill, E., 2020: RH-141455: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Limited, UK, Report No. 3202381, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	Only some minor deviations during the range-finder, which are considered to be not relevant for the integrity of the (main) study results.
GLP	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202716-01V, SMV 3202380-01V, and SMV 3202381-01V and following versions were used to determine the concentration of the analyte RH-141455 in samples of test media from acute fish, *Daphnia* and mysid studies, respectively. This method describes the analysis in Elendt M4 medium, treated mains water and in brackish water. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

For the determination of RH-141455 in treated mains water (fish study), samples were treated with acetonitrile containing 0.2% formic acid, then diluted further with acetonitrile/treated mains water (1:1 v/v) containing 0.1% formic acid as required to bring the response within the calibration range.

During the *Daphnia* study, concentrations of RH-141455 were determined by treating Elendt M4 medium samples with acetonitrile containing formic acid, then diluting further with Diluent 2 (acetonitrile/Elendt M4 medium 1:4 v/v containing 0.1% formic acid) as required to bring the response within the calibration range.

During the mysid study, concentrations of RH-141455 were determined by treating brackish water samples with milli-Q water/acetonitrile 8:2 v/v containing 0.1% formic acid, then diluting further with Diluent 2 (Brackish Water/Milli-Q Water/Acetonitrile 1:8:2 v/v, containing 0.1% formic acid) as required to bring the response within the calibration range.

Aliquots of the samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) system.

Equipment for LC-TOF/MS analysis

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Waters Acquity BEH phenyl, 1.7 µm, 50 x 2.1 mm (mysid) Scherzo SM-C18, 3.0 µm, 50 x 2.0 mm (fish, *Daphnia*)

Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 1% Formic acid in acetonitrile (LC-MS grade)

Fish acute toxicity test

Time [min]	A [%]	B [%]
0.01	95	5
3.00	95	5
3.90	0	100
4.00	0	100
5.00	95	5

Acute toxicity to *Daphnia*

Time [min]	A [%]	B [%]
0.01	95	5
1.00	95	5
3.00	0	95
3.90	0	95
4.00	95	5
5.00	95	5

Mysid acute toxicity test

Time [min]	A [%]	B [%]
0.01	95	5
1.00	95	5
3.00	10	90
3.90	10	90
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 50 µL (mysid, *Daphnia*), 25 (fish)
Retention time: approx. 3.9 min
Analysis time: 5 min
Ionisation: Electrospray (ESI), negativ
Ion mode: Product of 232.9403 Da : 188.928-188.978 Da

Results and discussions

Table A 46: Summary of recovery experiments - fish

Treated mains water	0.1 µg/mL	1 µg/mL	150 µg/mL	Overall
Mean Recovery (%)	113	107	102	108
CV (%)	2.4	0.94	1.3	4.4

Table A 47: Summary of recovery experiments - *Daphnia*

Elendt	0.1 µg/mL	10 µg/mL	150 µg/mL	Overall
Mean Recovery (%)	120	117	102	113
CV (%)	3.4	6.9	6.1	8.8

Table A 48: Summary of recovery experiments - mysid

Brackish water	0.25 µg/mL	1 µg/mL	150 µgm/L	Overall

Mean Recovery (%)	90.4	93.9	113	98.2
CV (%)	8.18	5.96	5.54	11.8

Accuracy and precision / repeatability

The accuracy and repeatability precision of the procedure were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response of the LC-TOF/MS analysis was determined over the concentration range 0.01 – 1 µg/mL (for fish), 0.002 – 0.5 µg/mL (for *Daphnia*) and 0.005 – 0.5 µg/mL (for mysid) for the respective media solutions. At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting. All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ was set to 0.1 mg/L (fish and *Daphnia*) and 0.25 mg/L (mysid).

Matrix effects

Matrix-matched standards were used.

Specificity

The LC-TOF/MS analysis method was considered specific as the analysis of the treated media exhibited no response exceeding 30% of the proposed LOQs.

Storage stability

Storage stability of frozen samples at ≤-10°C was demonstrated over a period of 8 days for fish samples, and over 29 days for *Daphnia* samples.

Table A 49: Characteristics of the analytical method validation for the determination of RH-141455 in aquatic samples

	RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r >0.99. Individual calibration data and calibration line equations presented in the study report
Calibration range	0.01 – 1 µg/mL (fish) 0.002 – 0.5 µg/mL (*Daphnia*) 0.005 – 0.5 µg/mL (mysid)
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ)	0.1 mg/L (fish and *Daphnia*) 0.25 µg/mL (mysid)

The following figure shows a typical chromatogram.

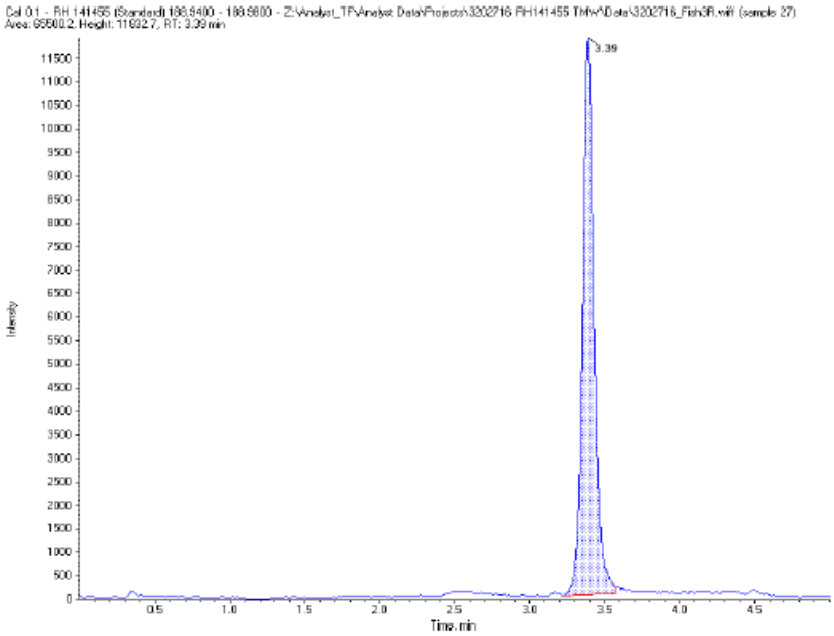


Figure A 23: **Chromatogram of RH-141455**

Conclusion

The analytical procedures for the determination of RH-141455 in aquatic media have been successfully validated according to SANCO 3029/99 rev 4.

(xxx 2020, xxx 2019, xxx. 2020)

A 2.1.1.6.6 Analytical method 6

The following method has been validated and used for the determination of RH-127450 in aquatic me-dia.

A 2.1.1.6.6.1 Method validation

Comments of zRMS:	The studies 5.1/39, 40, 41 are zoxamide confirmatory studies currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/39**
Report	xxx, 2020: RH-127450: Fish, acute toxicity test Gowan Crop Protection Ltd., UK xxx, UK, Report No. 3202373, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/40**

Report	Hugill, E., 2019: RH-127450: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202374, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	During the range finding test, the salinity of the control and highest concentration (100% saturated solution) exceeded the ± 1 ppt protocol requirement, as these were measured to be 22 and 18 ppt, respectively at the start of the test. This was not identified at the time in error, however, has no impact as the protocol requirement was tighter than the guideline (± 2 ppt). Additional dissolved oxygen concentrations were taken from the treatment vessels where 100% mortality was noted at the Study Director's direction. These were taken to confirm that the high mortality was not related to low oxygen levels, as mysids are very sensitive to oxygen levels. However, this protocol deviation has no impact on the integrity of the study. The protocol only required statistical analysis to be conducted on the 48 and 96-hour results, but should have included the conduct of statistical analysis on the 24 and 72-hour data, as this is a requirement of the OCSPP guideline.
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/41**
Report	Hugill, E., 2019: RH-127450: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ESG Ltd., Report No. 3202375, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	An initial definitive test was conducted at nominal concentrations of 1, 3.2, 10, 32 and 100% saturated solution. However, it was necessary to repeat the main test due to an equipment failure (lux meter) and since, by error, 24- and 48-hour samples were not taken for analysis. The appearance of the test item in the test media was only observed at the start and at the end of the test. This deviation has no impact on the integrity of the study as the non-inoculated media observation at 72-hours showed the test substance to be in solution.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202373-01V, SMV 3202374-01V and SMV 3202375-01V and following versions were used to determine the concentration of the analyte RH-127450 in samples of test media from acute fish, acute mysid and alga growth studies, respectively. The method describes the analysis of RH-127450 in mains water, brackish water and OECD medium. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

In the fish study, concentrations of RH-127450 were determined by treating samples with acetonitrile containing 0.5% formic acid, then diluting further with treated mains water/acetonitrile (mains water: acetonitrile 4:1, v/v, containing 0.1% formic acid) as required to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) system or a liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system.

In the mysid study, concentrations of RH-127450 were determined by treating brackish water samples with acetonitrile containing formic acid, then diluting further with Diluent 2 (Brackish water: acetonitrile 4:1, v/v, containing 0.1% formic acid) as required to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) system or liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system.

In the alga growth study, concentrations of RH-127450 were determined by treating OECD medium samples with acetonitrile containing formic acid, then diluting further with Diluent 2 (OECD medium: acetonitrile 4:1, v/v, containing 0.1% formic acid) as required to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) system.

RH-127450 is a racemate. In the fish study, an analytical method validation for the enantiomeric ratio analysis of RH-127450 was conducted under Smithers analytical procedure no. SMV 3202373-01V.E by inclusion of an enantioselective column. A combination of the above-mentioned analytical procedures with SMV 3202586 was used to assess in addition the enantiomeric ratio of the test substance in the test media and calibration standard solution.

Equipment for LC-TOF/MS analysis

Instrument:	AB Sciex TripleTOF5600+ coupled to Shimadzu SIL-30ACMP Quaternary HPLC system Analyst TF 1.7.1 data collection software
Column:	Waters Acquity BEH Phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase:	A: 0.1% Formic acid in water (LC-MS grade) B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	8 µL (mysid), 15 µL (alga), 5 µL (fish)
Retention time:	Approx. 2.15 min
Analysis time:	5 min
Ionisation:	Electrospray (ESI), positive
Ion mode:	Product of 302.07 Da : 186.941-186.991 Da

Equipment for LC-TQMS Analysis

Instrument:	API 5000 TQMS coupled to Shimadzu SIL-30ACMP Quaternary HPLC (mysid, fish) Agilent 1100 HPLC system with UV detector or Quaternary HPLC

Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.5 mL/min
Column temp.: 50°C
Injection volume: 3 µL (mysid), 5 µL (fish)
Retention time: 2.35 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 302 → m/z 187; m/z 302 → m/z 98

Results and discussions

Table A 50: Summary of recovery experiments - mysid

Tretend brackisch water	0.01 µg/mL	1 µg/mL	10 µg/mL	Overall
	LC-TOF/MS			
Mean Recovery (%)	112	104	86.1	101
CV (%)	0.98	3.7	2.0	12
	LC-TQMS			
Mean Recovery (%)	101	95.1	90.3	95.5
CV (%)	3.1	1.6	1.1	5.1

Table A 51: Summary of recovery experiments - alga

OECD medium	0.01 µg/mL	0.5 µg/mL	12 µg/mL	Overall
	LC-TOF/MS			
Mean Recovery (%)	106	101	97.3	101
CV (%)	3.80	5.55	4.82	5.68

CV Coefficient of variation

Table A 52: Summary of recovery experiments – fish

Tretend main water	0.001 µg/mL	1 µg/mL	150 µg/mL	Overall
	LC-TOF/MS			
Mean Recovery (%)	112	107	83.1	101
CV (%)	4.0	1.4	5.4	14
	LC-TQMS			

Mean Recovery (%)	101	99.6	95.9	98.8
CV (%)	3.4	4.0	2.2	3.7

CV Coefficient of variation

Accuracy and precision / repeatability

The accuracy and repeatability / precision of the procedure were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium (besides the overall RSD for LC/TOF-MS for fish with RSD = 14 => second method). Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response ~~of the LC-TQMS analysis~~ for RH-127450 was determined over the concentration range 0.0001 – 0.1 µg/mL for mysid and fish and 0.001 – 0.1 µg/mL for alga for the respective media solutions.

At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting.

All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ of the procedure is 0.01 mg/L (alga, mysid) and 0.001 mg/L (fish).

Matrix effects

Matrix-matched standards were used.

Specificity

Both methods were considered specific as the analysis of the treated media exhibited no response exceeding 30% of the proposed LOQ.

Storage stability

Storage stability of frozen samples at ≤-10°C was demonstrated over a period of 9 days for mysid samples, over 31 days for the fish study, and over 6 days for alga. Refrigerated extract stability was assessed at 2-8°C for a period of 7 days and met acceptance criteria.

Table A 52: Characteristics of the analytical method validation for the determination of RH-127450 in aquatic samples

	RH-127450
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r >0.99. Individual calibration data and calibration line equations presented in the study reports.
Calibration range	0.0001 – 0.1 µg/ml (mysid, fish) 0.001 – 0.1 µg/ml (alga)

Assessment of matrix effects is presented	No (matrix matched standard solutions).
Limit of quantification (LOQ)	LOQ: 0.01 mg/L (mysid, alga), 0.001 mg/L (fish)

The following figure shows a typical chromatogram.

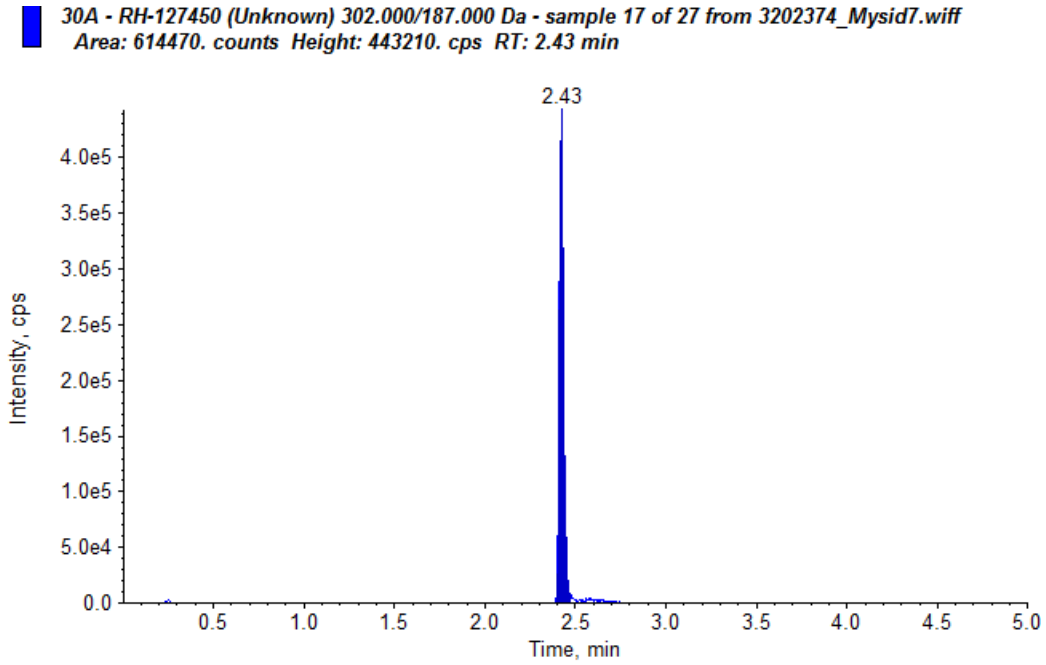


Figure A 24: **Chromatogram of RH-127450 (6.25% saturated solution test sample at 96 hours; dilution factor = 31.25)**

Conclusion

The analytical procedures for the determination of RH-127450 in aquatic media have been successfully validated according to SANCO 3029/99 rev 4.

(xxx 2019 a,b)

(xxx 2020)

A 2.1.1.6.7 Analytical method 7

A 2.1.1.6.7.1 Method validation

Comments of zRMS:	The study is confirmatory for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/42**
Report	Hugill, E., 2019: RH-24549: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202394, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4
Deviations:	The test media was prepared using reverse osmosis water rather than deionised water as per the definitive protocol. This protocol deviation has no impact on the integrity of the study because reverse osmosis water is acceptable according to

OCSPP 850.1035.
The salinity in the highest concentration dropped to 18 ppt (protocol requirement = 20±1 ppt during the test). This protocol deviation has no impact on the integrity of the study because salinity at 20±2 ppt is acceptable according to OCSPP 850.1035.
The protocol required statistical analysis to be conducted on the 48 and 96-hour results. However, it should have included additional statistical analysis on the 24 and 72-hour data. This protocol deviation has no impact on the integrity of the study since the necessary calculations have been performed.
A protocol deviation occurred at the highest concentration, because at 0-hours the pH in one test vessel was below pH 7.5 (allowed range: pH 7.5-8.5). This pH deviation was considered to have not an impact on the integrity of the study, because the 50% lethal effects concentration was between 25-50 mg/L where the pH measurements were within the acceptable range.

Acceptability: Yes

Materials and methods

The analytical procedure SMV 3202394-02V (later updated to SMV 3202394-05V to include stability data and corrects some typographical errors) was used to determine RH-24549 in aquatic samples of a mysid study. The method describes the analysis of RH-127450 in brackish water. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-24549 were determined by treating brackish water samples with acetonitrile containing formic acid, then diluting further with Diluent 2 (brackish water: acetonitrile 4:1, v/v, containing 0.1% formic acid) as required to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) or triple quad mass spectrometry (LC-TQMS/MS) system.

Equipment for LC-TOF/MS Analysis

Instrument: AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column: Waters Acquity BEH phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 ml/min
Column temp.: 50°C
Injection volume: 10 µL
Retention time: 2.16 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), negative

Ion mode: m/z 202.97 → m/z 158.953-159.003

Equipment for LC-TQMS Analysis

Instrument: AB Sciex API5000 TQMS Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.6.2 data collection software
Column: Waters Acquity BEH phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.5 ml/min
Column temp.: 50°C
Injection volume: 10 µL
Retention time: 2.25 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), negative
Ion mode: m/z 202.90 → m/z 158.90

Results and discussions

Table A 53: Summary of recovery experiments

Treated mains water	0.01 µg/mL	1µg/mL	150 µgm/L	Overall
	LC-TOF/MS			
Mean Recovery (%)	103.1	97.3	102.7	101
CV (%)	5.2	2.6	3.4	4.5
	LC- TQMS			
Mean Recovery (%)	100.4	95.6	99.7	98.6
CV (%)	2.3	4.3	4.3	4.1

Accuracy and precision/ repeatability

The accuracy and repeatability precision of the procedures were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ.
Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response of the LC-TOF/MS and LC-TQMS analysis for RH-24549 was determined over the concentration range 0.0005 – 0.1 µg/mL using calibration solutions prepared in diluent. At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all

greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting. All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ of the procedure is 0.1 mg/L.

Matrix effects

Matrix-matched standards were used.

Specificity

Both methods were considered specific as the analysis of the treated media exhibited no response exceeding 30% of the proposed LOQs.

Storage stability

Stability of fortified samples was confirmed at ≤-10°C over a period of 8 days.

Table A 54: Characteristics of the analytical method for the determination of RH-24549 in aquatic medium

	RH-24549
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r >0.99. Individual calibration data and calibration line equations presented in the study report
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix-matched standard calibration).
Limit of quantification (LOQ)	LOQ: 0.1 mg/L (0.1 µg/mL)

The following figure shows typical chromatogram

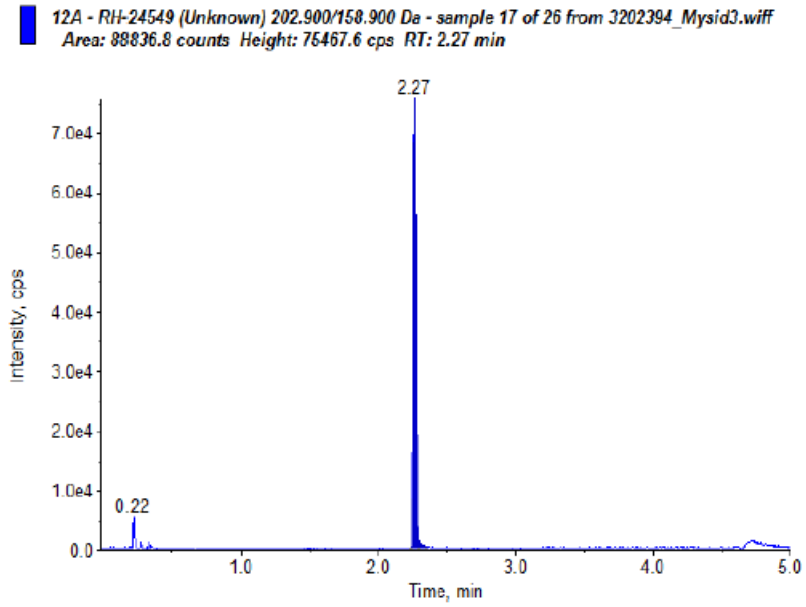


Figure A 25: **Chromatogram of RH-24549**

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev.4.

(Hugill E. 2019)

A 2.1.1.6.8 Analytical method 8

A 2.1.1.6.8.1 Method validation

Comments of zRMS:	The study is confirmatory for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/43**
Report	Hugill, E., 2019: RH-139432: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202398, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	The test media was prepared using reverse osmosis water rather than deionised water as per the definitive protocol. This protocol deviation has no impact on the integrity of the study because reverse osmosis water is acceptable according to OCSPP 850.1035. The study protocol requested statistical analysis only for the 48 and 96-hour results. However, according to OCSPP the protocol should also have been mentioned statistical evaluation of the 24 and 72-hour data, what has finally been performed and reported.
Acceptability:	Yes

Materials and methods

An analytical method (SMV 3202398-02V) for the determination of RH-139432 by LC-TOF in aquatic media (i.e. brackish water) has been developed and validated according to SANCO 3029/99 rev. 4.

Concentrations of RH-139432 were determined by treating brackish water samples with acetonitrile containing 0.5% formic acid, then diluting further with Diluent 2 (brackish water: acetonitrile 4:1, v/v, containing 0.1% formic acid) as required to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) or triple quadrupole mass spectrometry (LC-TQMS) system.

Equipment for LC-TOF/MS Analysis

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Waters Acquity BEH phenyl, 1.7 µm, 50 x 2.1 mm

Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 ml/min
Column temp.: 50°C
Injection volume: 10 µL
Retention time: 2.05 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 203.998 → m/z 186.947-186.997

Equipment for LC-TQMS Analysis

Instrument: AB Sciex API5000 TQMS Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.6.2 data collection software
Column: Waters Acquity BEH phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.5 mL/min
Column temp.: 50°C
Injection volume: 5 µL
Retention time: 2.10 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 204.1 → m/z 187.0

Results and discussions

Table A 55: Summary of recovery experiments

Brackish water	0.1 µg/mL	5µg/mL	50 µgm/L	Overall
	LC-TOF/MS			
Mean Recovery (%)	100	101	98.7	100
CV (%)	2.0	1.8	1.3	2.0
	LC-TQMS			

Mean Recovery (%)	104	98.2	92.7	98.2
CV (%)	2.9	1.1	2.7	5.2

Accuracy and precision/ repeatability

The accuracy and repeatability precision of the procedure were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ.
Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response of the LC-TOF/MS and LC-TQMS analysis for RH-139432 was determined over the concentration range 0.0001 – 0.1 µg/ml using calibration solutions prepared in diluent.

At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting.

All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ of the procedure is 0.1 mg/L.

Matrix effects

Matrix-matched standards were used.

Specificity

Both analysis methods were considered specific as the analysis of the treated media exhibited no response exceeding 30% of the proposed LOQs.

Storage stability

Stability of fortified samples was confirmed at ≤-10°C over a period of 8 days.

Table A 56: Characteristics of the analytical method for the determination of RH-139432 in aquatic media

	RH-139432
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r >0.99. Individual calibration data and calibration line equations presented in the study report.
Calibration range	0.0001 – 0.1 µg/ml
Assessment of matrix effects is presented	No (matrix-matched standard solutions).
Limit of quantification (LOQ)	LOQ: 0.1 mg/L

The following figure shows a typical chromatogram.

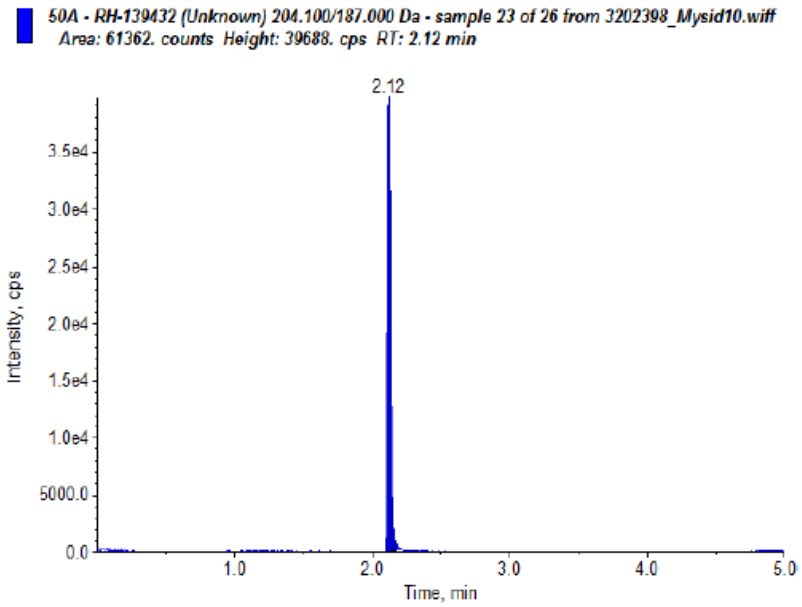


Figure A 26: **Chromatogram of RH-139432**

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4.

(Hugill E. 2019)

A 2.1.1.6.9 Analytical method 9

A 2.1.1.6.9.1 Method validation

Comments of zRMS:	The study is confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/44**
Report	Juckeland, D., 2019: Effects of zoxamide technical on *Lemna gibba* in a growth inhibition test under semi-static test conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 18 48 ALE 0005, 18 35 CRA 0021, GLP, Not published
Guideline(s):	SANCO/3029/99 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

A reverse phase-high performance liquid chromatography (RP-HPLC) method with mass spectrometric detection has been validated according to SANCO/3029/99 (2000) for the determination of zoxamide and its enantiomer's ratio in media of a *Lemna* toxicity test (BioChem project No. 18 48 ALE 0005).

The test medium was chosen according to OECD guideline, DMF served as solvent for zoxamide. After homogenisation of the specimens, the aquatic medium was homogenised and diluted 1:1 (v/v) with methanol and analysed.

After homogenization of the specimens, the aquatic medium/methanol was diluted and analysed.

Equipment

A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used.

Pumps:	LC-20ADXR
Degasser:	DGU-20A3R
Autosampler:	SIL-20ACXR
Column oven:	CTO-20AC
MS detector:	LCMS-8040
Controller:	CBM-20A
Data System:	LabSolutions Version 5.86
Column:	Lux 3µ Cellulose-3, 150 x 2 mm
Mobile phase:	A: Water with 5 mM ammonium formate and 0.1% (v/v) formic acid B: Methanol with 5 mM ammonium formate and 0.1% (v/v) formic acid
Gradient:	0.00 min: 60% B 7.00 min: 100% B 9.00 min: 100% B 9.01 min: 60% B 11.00 min: Stopp
Flow rate:	0.4 mL/min
Detection:	ESI positive, MRM, Zoxamide: m/z: 336 → 187, 336 → 159

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 57: Summary of recovery experiments

Validation	Replicates	Dilution factor	nominal conc. [µg/L]	measured conc. [µg/L]	recovery [%]	RSD [%]
Low	5	2	0.168	0.170	101	9.1
High	5	13.33	292.9	298.0	102	2.4

Accuracy and precision / repeatability

The recovery and precision data show that the influences of test medium when diluted 1:1 with methanol are for all analytes within the limits of the guidance document SANCO/3029/99; all criteria are fulfilled:

- blank values do not exceed 30% of the lowest validated concentration,
- mean recoveries for each level are in the range 70-110%,
- the RSD is < 20% per level.

No zoxamide was detected in the control solution.

Specificity

The method is regarded as specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as zoxamide, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ). Example LC-MS/MS chromatograms of external standard, control (untreated alga medium and tissue) and fortified matrix solutions are presented in the report.

Linearity

Linearity was demonstrated for (RS)-zoxamide and for both isomers. The calibration ranges were between 0.055 – 26.48 µg/L with 9 concentration levels for matrix-matched calibration curve. This covers ranges from at least 30 % of the LOQ to at least 20 % above the highest concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this analytical phase as the lowest successfully validated fortification level, i.e. 0.084 µg/L (RS)-zoxamide in samples diluted 1:1 (v/v) with methanol, this corresponds to 0.168 µg/L (RS)-zoxamide in undiluted test solutions.

Limit of detection

The limit of detection (LOD) was defined in the context of this analytical phase as the lowest measured calibration concentration, i.e. 0.055 µg/L (RS)-zoxamide.

Matrix effects

Matrix effects were not investigated. Matrix standards were used for quantification of zoxamide.

Storage stability

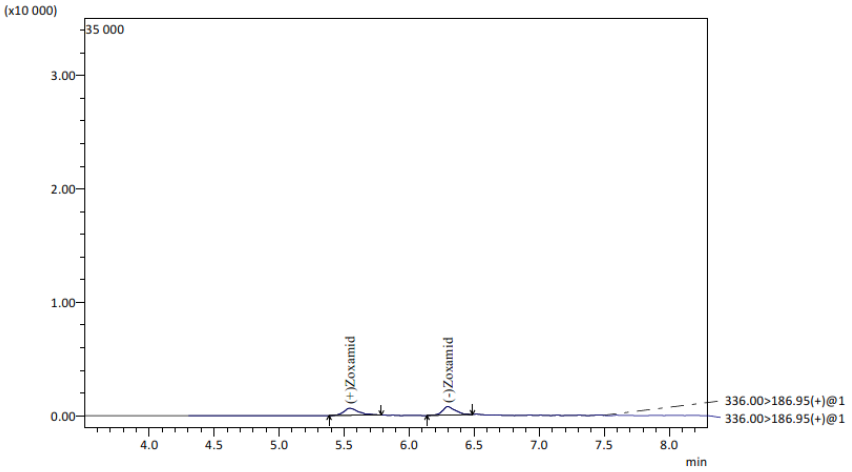
Stability was not investigated. Moreover, there were no sample extracts or solvent standards that were stored for > 24 hours in the fridge (at about 4°C). The maximum storage time of deep-frozen test samples (at ≤ 18°C) was 27 day (< 30 days).

Table A 58: Characteristics of the analytical method for the determination of zoxamide in aquatic medium

	Zoxamide
Specificity	Chiral HPLC with MS/MS detection monitoring two ion mass transitions for quantification and/or qualification characteristic retention time of the analytes; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	9-point calibration with external standard ; linear; r >0.99 Calibration curve equations: y = 94584.4 x + 4940.64, r = 0.9999589
Calibration range	0.055 to 26.48 µg/L in analytical samples
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had no detectable peaks. No interfering peaks (< 30 % LOQ) were detected.
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.168 µg/L for zoxamide LOD: 0.055 µg/L for zoxamide

The following figures show typical chromatograms.

Sample Name:	HTA21-Cal 1	Vial#	2
Sample Type:	Standard	Injection Volume:	10 µL
Acquired:	06.09.2018 16:21:55	Processed:	13.09.2018 07:18:26
Data File:	HT_002.lcd	Method File:	Zox_HT.lcm



Quantitative Results

ID#	Name	Ret. Time	m/z	Area	Conc.	Unit	Ref.1 m/z	Ref.1 Act%
1	(+)Zoxamid	5.542	336.00>186.95	4918	0.028	µg/L	336.00>159.00	61.39
2	(-)Zoxamid	6.297	336.00>186.95	5027	0.025	µg/L	336.00>159.00	61.70
Total				9946	0.053			

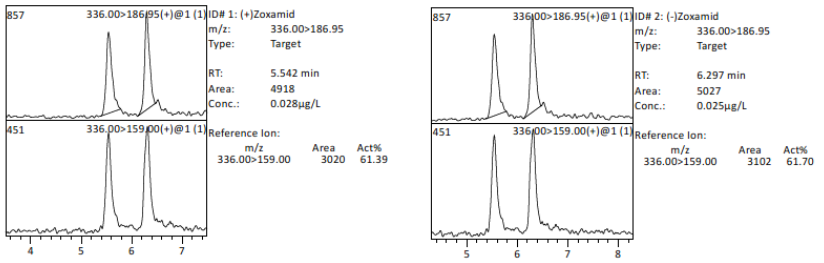
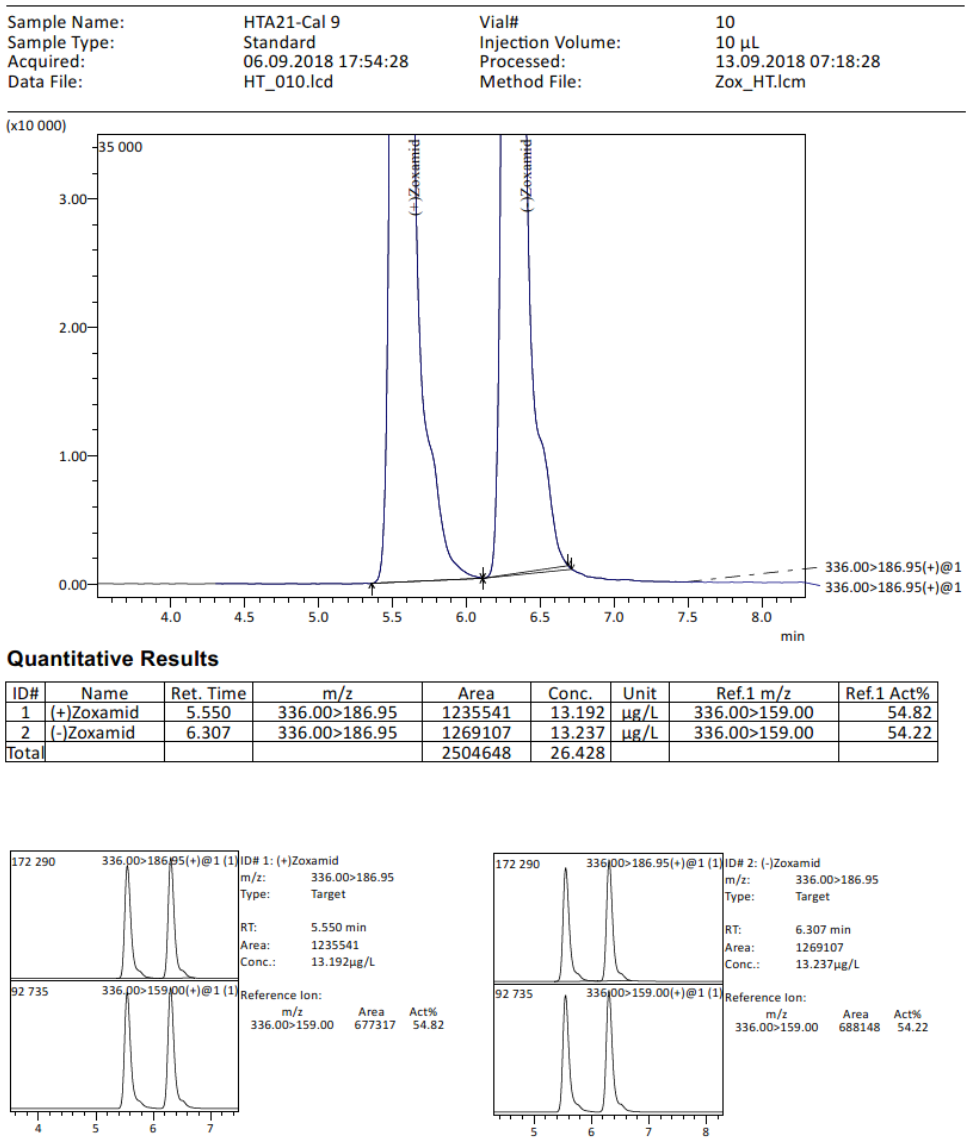


Figure A 27: Chromatogram of the lowest calibration standard



Quantitative Results

ID#	Name	Ret. Time	m/z	Area	Conc.	Unit	Ref.1 m/z	Ref.1 Act%
1	(+)Zoxamid	5.550	336.00>186.95	1235541	13.192	µg/L	336.00>159.00	54.82
2	(-)Zoxamid	6.307	336.00>186.95	1269107	13.237	µg/L	336.00>159.00	54.22
Total				2504648	26.428			

Figure A 28: Chromatogram of the highest calibration standard

The enantiomeric ratio analysis confirmed the stability of the chiral C of zoxamide – it stayed in the test media as a racemate over the test periods. Results are summarised in the following table.

Table A 59: Enantiomeric ratios

Samples	Mean enantiomeric ratio
Calibration	1.04
Validation low	1.05
Validation high	1.05
Treatment 9	1.05
Treatment 8	1.00
Treatment 7	1.01
Treatment 6	1.06
Treatment 5	1.00
Treatment 4	1.07
Treatment 3	1.07

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4.

The enantiomeric ratio of the test item is slightly different from 1 (1.00 to 1.07) but close enough to 1 to state that zoxamide existed as the racemate in the specimens.

(Juckeland D. 2020)

A 2.1.1.6.10 Analytical method 10 – method for the determination of zoxamide and cymoxanil in aqueous media (34-98-5)

A 2.1.1.6.10.1 Method validation

Comments of zRMS:	The study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/45**
Report	xxx, 2007: Cymoxanil 33% + Zoxamide 33% WG: validation of the analytical method for the determination of the content of zoxamide in water samples from the aquatic ecotoxicological studies Oxon Italia S.p.A., Italy xxx., Report No. CH-156/2006, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANCO/825/00 rev. 7 (2004)
Deviations:	None
Acceptability:	Yes

Principle of the method

Residues of zoxamide in water samples from the aquatic ecotoxicological studies performed were determined in GC/MS technique, in SIM mode (DAS method 34 – 98 – 52).
Repeatability and recovery tests were performed on samples at 10 µg/L, 100 µg/L, 300 µg/L and 330 mg/L of zoxamide nominal level. Eight samples were analysed for each concentration.

GC/MS parameters	
Column	ChemService code No. 139 (Supelco or equivalent: SLB - %MS 30 m x 0.25 mm, film thickness 0.25 µm)
Column temperature	5°C / min from 150 °C to 250 °C, 20 °C / min from 250 to 300 °C, 2 min at 300 °C
Injector temperature	250 °C
Carrier flow	1 ml/ min He
Volume of injection	1 µl; splitless 2 min
Source temperature	250 °C

Results

Specificity
The method was demonstrated to be specific and sensitive for determination of zoxamide residues in water samples by GC/MS technique in SIM mode. zoxamide was identified on the basis of fragmentation ions

187, 242, and 258 m/z, at two different retention times (13.183 and 18.700 min). The presence of Zoxamide in water samples was determined by comparing the two peaks characteristic for the active ingredient with those obtained for analytical standard solutions.
No interferences were also observed.

Linearity
The linearity was checked on a range of concentration from 20 to 400 ng/ml.

Accuracy
The accuracy of the analytical method was considered to be acceptable, since the mean recovery for all fortifications was in the range of 88 – 100%.

Repeatability
The RSD for each level of fortification is lower than 20%, so the precision of the analytical method can be considered acceptable.

In conclusion, the analytical method was regarded as suitable for the quantification of zoxamide in water samples at an LOQ of 20 ng/mL.

(xxx 2007)

A 2.1.1.6.10.2 Method validation

The above method (34-98-5) has been applied in the following study.

Comments of zRMS:	The study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/46**
Report	xxx, 2007: Acute toxicity of Cymoxanil 33% + Zoxamide 33% WG to rainbow trout (*Oncorhynchus mykiss*), determined under flow-through conditions. Oxon Italia S.p.A., Italy xxx, Report No. CH-E–023/2006, GLP, Not published
Guideline(s):	OECD 203 (1992) EU Commission Directive 92/69/EEC, C1 (1992) OPPTS 850.1075 (1996)
Deviations:	No
GLP:	Yes
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

ChemService S.r.I. conducted a study to determine the test article content in water samples coming from the ecotoxicological tests performed by ChemService Ecotox Department.

The study was performed in accordance with analytical phase Study Protocol CH-157/2006 and was fully validated according to SANCO guidance's 3029/99 rev. 4 and 825/00 rev. 7.

In a preliminary non GLP phase (study No. CH-156/2006), it was found that injecting standard and water extract samples, two major peaks were obtained in GC/MS analysis, each of them attributed to zoxamide

because of the presence of characteristic ions. After several tests with different injector temperatures and taking into consideration that the high grade certified analytical standard eluted in these two peaks, it was finally supposed that there a probable degradation of the active ingredient in the heated liner into the split-splitless injector.

To avoid or at least to limit this degradation without a contemporary loss of sensitivity, as best compromise condition was chosen to take into consideration the contribution of both peaks by a sum of the peak areas of the common ion at 187 m/z using the selected ions monitoring (SIM) technique.

The samples were stored at 4°C for 30 days.

Equipment

Instrument:	Gas chromatograph Thermo Finnigan Mod. Trace GCUltra, equipped with split/splitless injector, autosampler AS3000, and coupled with a Thermo Finnigan Polaris Q ion trap mass detector

For further MS conditions, please refer to the study report.

Results and discussions

Table A 60: Summary results

ChemService No.	Time	Type (*)	Zoxamide (µg/L)	Formulation (µg/L)	Recovery vs. nominal
AA29019	0 h	Control	n.d.	-	-
AA29020	0 h	Test concentration 0.10 mg/L	16.02	48.55	48.5%
AA29021	0 h	Test concentration 0.32 mg/L	71.10	215.45	67.3%
AA29022	0 h	Test concentration 1.00 mg/L	106.87	323.85	32.4%
AA29023	0 h	Test concentration 3.20 mg/L	188.02	569.76	17.8%
AA29024	0 h	Test concentration 10.00 mg/L	294.23	891.61	8.9%
AA29025	48 h	Control	n.d.	-	-
AA29026	48 h	Test concentration 0.10 mg/L	29.20	88.48	88.5%
AA29027	48 h	Test concentration 0.32 mg/L	69.58	210.85	65.9%
AA29028	48 h	Test concentration 1.00 mg/L	118.92	360.36	36.0%
AA29029	48 h	Test concentration 3.20 mg/L	163.36	495.03	15.5%
AA29030	48 h	Test concentration 10.00 mg/L	237.39	719.36	7.2%
AA29031	72 h	Test concentration 10.00 mg/L	209.94	636.18	6.4%
AA29032	96 h	Control	n.d.	-	-
AA29033	96 h	Test concentration 0.10 mg/L	19.53	59.18	59.2%
AA29034	96 h	Test concentration 0.32 mg/L	68.06	206.24	64.5%
AA29035	96 h	Test concentration 1.00 mg/L	124.98	378.73	37.9%
AA29036	96 h	Test concentration 3.20 mg/L	155.11	470.03	14.7%

* The nominal concentration for ecotoxicological fortification samples is expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.

Accuracy and precision / repeatability

For accuracy, the SANCO guideline requires mean recovery values in the range from 70 to 110% at each level; the slight deviation obtained can be accepted because of the low water solubility of the test substance; therefore, the accuracy of the analytical method was considered to be acceptable.

For the precision, the SANCO guideline requires an RSD % lower than 20 % for each level.

Linearity

The range tested was from 20 to 400 ng/ml, corresponding to zoxamide concentrations from 10 to 200 µg/L in the water samples, was found to be linear (correlation coefficient > 0.99).

Limit of quantification

The limit of quantification (LOQ) of this method is defined as the lowest fortification level for which recovery in the range of 70-110 % and CC < 20 % are obtained, was found to be of 10 µg/L in water matrix samples, corresponding to a final solution of 20 ng/ml (ppb).

Limit of detection

The limit of detection (LOD) of this method is defined as 50% of the lowest calibration level, i.e. 10 ng/ml (ppb) corresponding to 5 µg/L in the water matrix samples.

Matrix effects

Matrix-matched standards were used.

Specificity

The GC MS method is regarded specific.

Storage stability

The samples were stored at 4°C for 30 days.

Table A 61: Characteristics of the analytical method validations for zoxamide in aquatic media

	Zoxamide
Specificity	The GC MS method is regarded specific Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	5-point calibration with external matrix-matched standard The calibration was linear. r^2 = 0.99990
Calibration range	10 to 200 µg/L
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 10 µg/L LOD: 5 µg/L

The following figures show typical chromatograms.

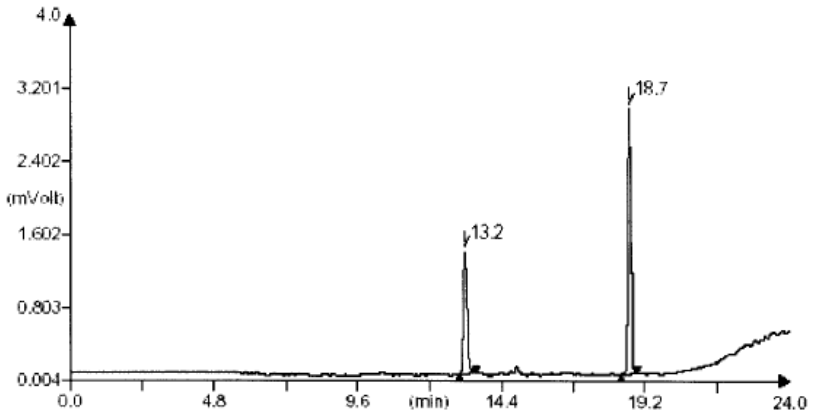


Figure A 29: **Representative chromatogram of the standard solution of zoxamide**

Conclusion

The analytical method was shown to be specific for zoxamide residues in water samples from the aquatic ecotoxicological studies.

(xxx 2007)

A 2.1.1.6.10.3 Method validation

The above method (34-98-5) has been applied in the following studies.

Comments of zRMS:	The studies 5.1/47 and 48 were already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/47**
Report	Croce, V., 2007: Acute toxicity of Cymoxanil 33% + Zoxamide 33% WG to *Daphnia magna* in a 48-hour immobilization test under semi-static exposure - limit test Oxon Italia S.p.A., Italy ChemService S.r.I., Italy, Report No. CH-E-001/2007, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000) SANCO 825/00 rev. 7
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/48**
Report	Croce, V., 2007: Toxicity of Cymoxanil 33% + Zoxamide 33% WG to green algae *Pseudokirchneriella subcapitata* determined in a growth inhibition study Oxon Italia S.p.A., Italy ChemService S.r.I., Italy, Report No. CH-E-002/2007, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)

SANCO 825/00 rev. 7

Deviations: No

Acceptability: Yes

Materials and methods

ChemService S.r.I. conducted a study to determine the test article content in water samples coming from the ecotoxicological tests performed by ChemService Ecotox Department.

All controls and fortified water samples were extracted and analysed following ChemService Analytical Method No. 156/2006. The method was fully validated according to SANCO 3029/99 rev. 4 and SANCO 825/00 rev. 7

In a preliminary non GLP phase (study No. CH-156/2006), it was found that injecting standard and water extract samples, two major peaks were obtained in GC/MS analysis, each of them attributed to zoxamide because of the presence of characteristic ions. After several tests with different injector temperatures and taking into consideration that the high grade certified analytical standard eluted in these two peaks, it was finally supposed that there a probable degradation of the active ingredient in the heat-ed liner into the split-splitless injector.

To avoid or at least to limit this degradation without a contemporary loss of sensitivity, as best com-promise condition was chosen to take into consideration the contribution of both peaks by a sum of the peak areas of the common ion at 187 m/z using the selected ions monitoring (SIM) technique.

The samples were stored at 4°C for 30 days.

Equipment

Instrument: Gas chromatograph Thermo Finnigan Mod. Trace GCUltra, equipped with split/splitless injector, autosampler AS3000, and coupled with a Thermo Finnigan Polaris Q ion trap mass detector

Results and discussions

Table A 62: Summary results in ecotoxicological water samples (daphnia magna)

ChemService No.	Time	Type (*)	Zoxamide (mg/L)	Formulation. (mg/L)	Recovery vs. nominal
AA35186	0 h	Control (time 0)	n.d.	-	-
AA35187	0 h	Test concentration 100 mg/L (time O)	22.69	68.7	69 %
AA35188	24 h old	Control (after 24h)	n.d.	-	-
AA35189	24 h old	Test concentration 100 mg/L (after 24h)	8.39	25.4	25 %
AA35190	24 h new	Control (new solution after 24 h)	n.d.	-	-
AA35191	24 h new	Test concentration 100 mg/L (new solution after 24 h)	22.97	69.6	70 %
AA35192	48 h	Control (after 48h)	n.d.	-	-
AA35193	48 h	Test concentration 100 mg/L (after 48h)	10.68	32.4	32 %

(*) The nominal concentration for ecotoxicological fortification samples are expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.
n.d. not detected, lower than LOD (5 µg/L)

Table A 63: Summary results in ecotoxicological water samples (green algae)

ChemService No.	Time	Type (*)	Zoxamide (mg/L)	Formulation (mg/L)	Recovery vs. nominal
AA35195	0 h	Control t0	n.d.	-	-
AA35196	0 h	Test concentration 0.03 mg/L	0.021	0.062	210
AA35197	0 h	Test concentration 0.07 mg/L	0.051	0.156	255
AA35198	0 h	Test concentration 0.17 mg/L	0.071	0.216	118
AA35199	0 h	Test concentration 0.41 mg/L	0.132	0.399	94
AA35200	0 h	Test concentration 1.00 mg/L	0.331	1.002	100
AA35201	72 h	Control t72h	n.d.	-	-
AA35202	72 h	Test concentration 0.03 mg/L	0.008	0.025	80
AA35203	72 h	Test concentration 0.07 mg/L	0.036	0.109	165
AA35204	72 h	Test concentration 0.17 mg/L	0.039	0.118	65
AA35205	72 h	Test concentration 0.41 mg/L	0.908	0.275	65
AA35206	72 h	Test concentration 1.00 mg/L	0.168	0.509	51
AA35207	72 h	Test concentration 0.03 mg/L WAI	0.042	0.127	170
AA35208	72 h	Test concentration 0.07 mg/L WAI	0.031	0.095	195
AA35209	72 h	Test concentration 0.17 mg/L WAI	0.057	0.171	95
AA35210	72 h	Test concentration 0.41 mg/L WAI	0.117	0.354	84
AA35211	72 h	Test concentration 1.00 mg/L WAI	0.216	0.654	65

(*) The nominal concentration for ecotoxicological fortification samples are expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.
n.d. not detected, lower than LOD (5 µg/L)
WAI: without algal inoculum.

Accuracy and precision / repeatability

For accuracy, the SANCO guideline requires mean recovery values in the range from 70 to 110% at each level; the slight deviation obtained can be accepted because of the low water solubility of the test substance; therefore, the accuracy of the analytical method was considered to be acceptable.

For the precision, the SANCO guideline requires an RSD % lower than 20 % for each level.

Linearity

The range tested was from 20 to 400 ng/ml, corresponding to zoxamide concentrations from 10 to 200 µg/L in the water samples, was found to be linear (correlation coefficient > 0.99).

Limit of quantification

The limit of quantification (LOQ) of this method is defined as the lowest fortification level for which recovery in the range of 70-110 % and CC < 20 % are obtained, was found to be of 10 µg/L in water matrix samples, corresponding to a final solution of 20 ng/ml (ppb).

Limit of detection

The limit of detection (LOD) of this method is defined as 50% of the lowest calibration level, i.e. 10 ng/ml (ppb) corresponding to 5 µg/L in the water matrix samples.

Matrix effects

Matrix-matched standards were used.

Specificity

The GC MS method is regarded specific.

Storage stability

The samples were stored at 4°C for 30 days.

Table A 64: Characteristics of the analytical method validations for zoxamide in aquatic media

	Zoxamide
Specificity	The GC MS method is regarded specific Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	5-point calibration with external matrix-matched standard The calibration was linear. r^2 = 0.99990
Calibration range	10 to 200 µg/L
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 10 µg/L LOD: 5 µg/L

The following figures show typical chromatograms.

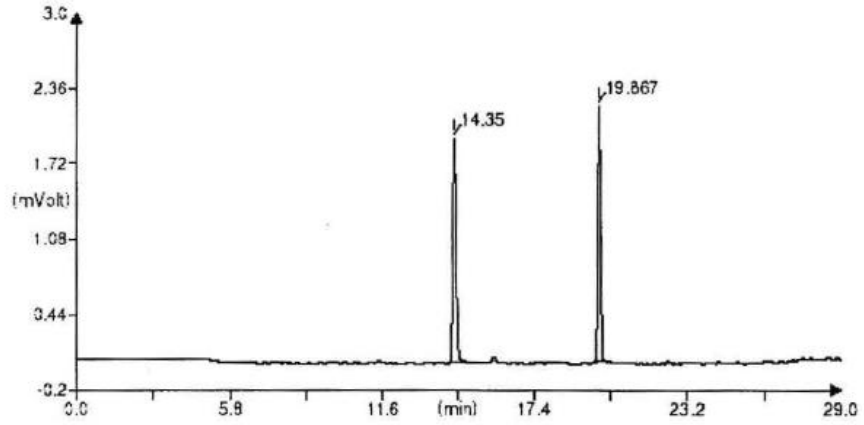


Figure A 30: **Representative chromatogram of the standard solution of zoxamide**

Conclusion

The analytical method was shown to be specific for zoxamide residues in water samples from the aquatic ecotoxicological studies.

(xxx. 2007)

A 2.1.1.6.11 Analytical method 11 – determination of zoxamide in salt water

A 2.1.1.6.11.1 Method validation

Comments of zRMS:	The studies 5.1/49, 50 are confirmatory studies for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/49**
Report	xxx., 2020: Final report addendum for RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) Gowan Crop Protection Ltd., UK xxx, Report No. 129A-143A, GLP, Not published
Guideline(s):	OCSPP 850.1400 (2016) SANCO 3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/50**
Report	xxx, 1998: RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) xxx, Report no. 97RC-0078 xxx, Amendment No. 129A-143A GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	Two sets of Day 0 analytical samples were collected. The first set of samples contained an interference in the chromatography. An additional set of samples was collected and analysed using a direct injection method which removed the interference. The protocol states that method validation which bracelets the concentrations of the study will be conducted and approved by Dr. Sandra Ferris prior to initiation of the definitive. The method was actually validated after test initiation since the first set of samples contained an interference in the chromatography. A new analytical method was developed concurrently with the Day 0 samples. However, formal validation of the method occurred after test initiation. The deviations did not adversely affect the results and the integrity of the study.
Acceptability:	Yes

Materials and methods

Zoxamide (RH-117,281) in saltwater samples of a fish early life-stage (ELS) study with sheepshead minnow (*Cyprinodon variegatus*) was analysed with a high-performance liquid chromatography (HPLC) combined with UV detection.

The method comprised a direct analysis of the aqueous samples, diluted with filtered saltwater as needed, and analysed by reverse-phase chromatography with UV detection at an LOQ of 0.01 mg a.i./L. Test item concentrations were calculated using linear regression from external matrix matched (filtered saltwater) standard solutions.

The method was validated with filtered saltwater spiked with the test item zoxamide at four concentrations of 0.005, 0.015, 0.100 and 0.400 mg/L. In addition, saltwater samples fortified at 0.015, 0.100 and 0.400 mg a.i./L were analysed concurrently with the test item samples to observe procedural recoveries during the test item measurements.

In an amendment to this study (xxx., 2020), the analytical method used to analyse test solution samples in the original study were assessed for compliance with current SANCO guidance. The specificity of the method (confirmatory method) and its repeatability, as well as the freezer storage stability of samples and the stability of sample extracts (in case storage of samples was required) were assessed based on review of the original raw data.

Equipment

Instrument: Hewlett-Packard Model 1090 High Performance Liquid Chromatograph with diode array detector (DAD) or a Waters 486 Tunable Absorbance Detector
Column: Phenomenex Inertsil ODS-2 (250 nun x 4.6 nun, 5, µm particle size)
Mobile phase: Solvent A: Water
Solvent B: Acetonitrile

Gradient:

Time (min)	%A	%B	Curve
0.01	70	30	--
1.00	70	30	Hold
10.0	0	100	Linear
12.0	0	100	Hold
12.1	70	30	Step
17.0	70	30	Hold

Flow rate: 1.00 mL/min
Injection volume: 200 µL
Stop time: 17 minutes
Oven temperature: 40°C
Retention time: Zoxamide (RH-117,281): 13 minutes

Results and discussions

Table A 65: Summary of recovery experiments from the original study report

Matrix	Fortification mg/L	Recovery	Mean recovery	Acceptable recovery	Acceptable RSD
Saltwater (fish medium)	0.005	107, 108, 108 %	108 %	70 – 110 %	20 %
	0.015	103, 104, 104 %	104 %		
	0.100	99.5, 99.8, 99.2 %	99.5 %		
	0.400	99.1, 100, 99.4	99.5 %		

During the study, matrix samples were fortified with RH-117,281 technical at 0.015, 0.100 and 0.400 mg a.i./L to determine recovery and to evaluate method performance. Summary information of the additional data is given in the following table.

Table A 66: Summary of recovery experiments from the amendment to the study report

Sample		Concentration RH-117,281 (mg a.i./L)		% recovery[2]	Mean measured[5] (mg a.i./L)	Mean % recovery ($\bar{x}$) SD RSD[5]
Number (129A-143A-)	Type	Fortified	Measured[12]			
MAB-2	Matrix Blank	0.0	< LOQ	-	-	-
MAB-3	Matrix Blank	0.0	< LOQ	-		
MAB-4	Matrix Blank	0.0	< LOQ	-		
MAB-5	Matrix Blank	0.0	< LOQ	-		
MAB-6	Matrix Blank	0.0	< LOQ	-		
MAB-7	Matrix Blank	0.0	< LOQ	-		

MAS-4	Matrix Fortification	0.015	0.0155	103	0.0149	$(\bar{x})$ = 99.4
MAS-7	Matrix Fortification	0.015	0.0169[3]	113[3]		SD = 8.95
MAS-10	Matrix Fortification	0.015	0.0142	94.8		RSD =
MAS-13	Matrix Fortification	0.015	0.0079[3]	53.2[4]		9.00%
MAS-16	Matrix Fortification	0.015	0.0144	96.1		
MAS-17	Matrix Fortification	0.015	0.0135	89.9		
MAS-5	Matrix Fortification	0.100	0.101	101	0.102	$(\bar{x})$ = 102
MAS-8	Matrix Fortification	0.100	0.106	106		SD = 2.14
MAS-11	Matrix Fortification	0.100	0.100	100		RSD =
						2.10%
MAS-14	Matrix Fortification	0.100	0.103	103		
MAS-17	Matrix Fortification	0.100	0.102	102		
MAS-20	Matrix Fortification	0.100	0.101	101		
MAS-6	Matrix Fortification	0.400	0.398	99.6	0.394	$(\bar{x})$ = 98.4
MAS-9	Matrix Fortification	0.400	0.374	93.6		SD = 2.53
MAS-12	Matrix Fortification	0.400	0.400	100		RSD =
						2.57%
MAS-15	Matrix Fortification	0.400	0.390	97.5		
MAS-18	Matrix Fortification	0.400	0.401	100		
MAS-21	Matrix Fortification	0.400	0.399	99.7		
			Mean[3] =	100		
			Standard Deviation[3] =	5.13		
			RSD[3] =	5.13%		

1 During the study the method limit of quantitation (LOQ) was set at 0.0100 mg a.i./L and was calculated as the product of the lowest standard (0.0100 mg a.i./L) and the dilution factor of the matrix blank (1.00). The SANCO method limit of quantitation (LOQ) was defined as 0.015 mg a.i./L, the lowest matrix fortification concentration in which a mean recovery of 70-110% was obtained.
2 Results were generated using Excel 4.0 V. Manual calculations may differ slightly.
3 The measured value was extrapolated below the curve.
4 The low recovery was likely due to a fortification error. This result has been excluded from the calculation of the mean as an outlier.
5 Results were generated using Microsoft Excel 2010. Manual calculations may differ slightly.

Accuracy and precision / repeatability

The method is considered acceptable according to SANCO/3029/99 rev. 4 for accuracy with mean recovery values in the range of 70 to 110% for each fortification level and for precision with relative standard deviations of ≤20% per level.

The precision of the method has been reported as the relative standard deviation (RSD) of repeatability at each fortification level and the overall RSD. In general, the RSD should be ≤20% per fortification level. The precision and repeatability criteria were met at the 0.015, 0.100 and 0.400 mg a.i./L matrix fortification levels.

Linearity

Calibration with matrix matched standard solutions (5 points) for zoxamide, with concentrations ranging from 0.010 to 0.200 mg a.i./L, were linear (r = 1.0). A representative calibration curve is presented in the study report.

Based on the amendment to the report, the calibration standard series were injected at the beginning and end of the analytical run with, in addition, a minimum of one standard injected following every five samples. One or more calibration curves were derived from regression analysis of the instrumental responses

of the standards as an assessment of linearity. The linear calibration range exceeded the expected nominal concentrations in final dilutions/extracts by at least ±20% for samples with test concentrations of 0.075, 0.15 and 0.30 mg a.i/L. This was not achieved for study samples 0.019, 0.038 and the low matrix fortification 0.015 mg a.i./L (LOQ during the study).

Limit of quantification

During the study the method limit of quantitation (LOQ) was set at 0.0100 mg a.i./L and was calculated as the product of the lowest standard (0.0100 mg a.i./L) and the dilution factor of the matrix blank (1.00). The SANCO/3029/99 rev. 4 method limit of quantitation (LOQ) is defined as 0.015 mg a.i./L, the lowest matrix fortification concentration for which a mean recovery of 70-110% was obtained.

Matrix effects

Matrix matched standards were used.

Specificity

No interferences > 30 % LOQ were observed at or above the LOQ at the retention time of interest for the blank matrix solutions (filtered salt water solutions), demonstrating the specificity of the method.

Table A 67: Characteristics of the analytical method for the determination of zoxamide in fish medium

	Zoxamide
Specificity	Typical chromatograms are provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	5 points calibration with matrix matched standard solutions. Correlation coefficient r > 0.99. A representative calibration curve and calibration line equation is presented in the study report.
Calibration range	0.0100 to 0.200 mg a.i./L (new: 0.015-0.200); linear
Assessment of matrix effects is presented	No (matrix matched standards used).
Limit of quantification (LOQ)	LOQ: 0.01 mg a.i./L (new: 0.015 mg a.i./L)

The following figure shows typical chromatogram

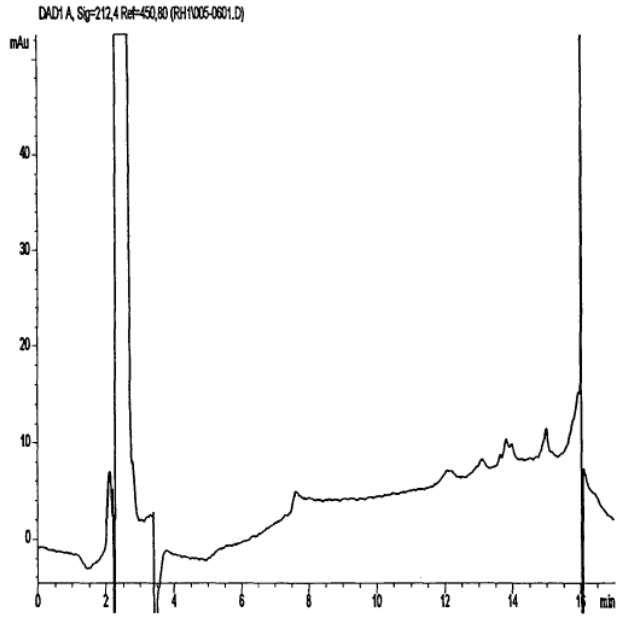


Figure A 31: A representative chromatogram of matrix blank

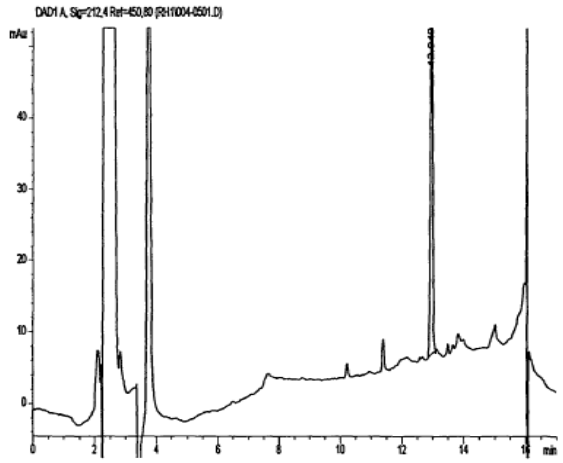


Figure A 32: A representative chromatogram of a 0.200 mg a.i./L RH-117,281 standard (40 ng on column)

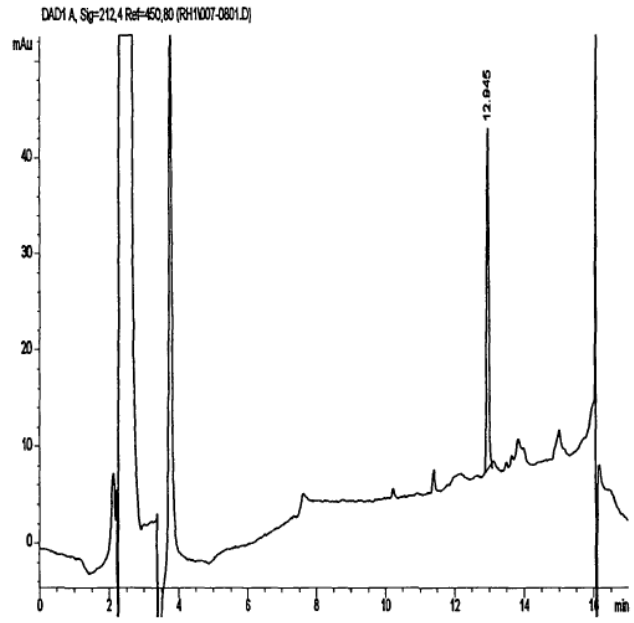


Figure A 33: A representative chromatogram of matrix fortification (0.100 mg a.i./L RH-117,281)

Conclusion

The analytical method for the determination of zoxamide in fish medium is regarded valid for the date of the time when the study has been performed. A re-evaluation according to current guidelines (SANCO/3029) confirmed this: The analytical findings met all data requirements with the exception of a confirmatory method.

(xxx 1998)

(xxx 2020)

A 2.1.1.7 Description of analytical methods for the determination of residues in soil

A 2.1.1.7.1 Analytical method 1

A 2.1.1.7.1.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/51**
Report	Kercher, S., 2017: Enantioselective degradation of (R)-zoxamide and (S)-zoxamide in one soil incubated under aerobic conditions Gowan Crop Protection Ltd., UK RLP AgroScience, Germany, Report No. AS520, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 6 (20.06.00) SANCO/3029/99 rev. 4 (11.07.00)
Deviations:	The temperature in the incubation chamber should be held at 20±2 °C

	throughout the study, but it decreased to below 18 °C (min 17.7 °C) on 1 occasion for a time period <1 day. The average temperature remained at 20.2±0.1 °C. This deviation is regarded to not alter the results of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study was performed with zoxamide (racemate) as test item. The enantioselective degradation of (R)- and (S)-zoxamide and its metabolites RH-127450 and RH-16653 was followed in one soil incubated under aerobic conditions and analysed by enantioselective liquid chromatography coupled with a tandem mass spectrometer detector.

Entire soil samples were extracted with 100 mL acetonitrile/water (100:2; v/v). The flasks were shaken for 30 minutes, the samples were centrifuged for 5 minutes at 2000 rpm, and the supernatants removed. The volumes were adjusted to 100 mL with water in volumetric flasks. Two aliquots per sample were taken, diluted with acetonitrile/water (1:1, v/v) with a factor of 100 and measured by LC-MS/MS. Each soil sample was extracted twice.

Soil

One loam soil used in this study was freshly sampled from an arable field site in "Mußbach", Germany, from the top layer (20 cm, no chemical treatments last 3 years).

Table A 68: Soil characteristics

Soil type	Source	pH (water)	Water holding capacity (g/100g)	Soil density (g/L)
Loam	Mußbach, Germany (Coordinates 49 22´5 74" N, 8 11´7 42" E)	7.6	31.53	1.5 g/cm^3 (nominal value)

Maximum water holding capacity at pF 2.0

Equipment method 1 – for the determination of zoxamide and RH-127450

Instrument:	Agilent Infinity II 1260 system equipped with Infinity II 1260 autosampler and Infinity II1260 pump AB Sciex 6500+ QTrap mass spectrometer, electron impact (EI) ion volume Analyst 1.6 LC/MS instrument control and MuliQuant 3.0.2 data acquisition software
Column:	250 mm x 4.6 mm, 5 µm Phenomenex LUX Cellulose-3
Column oven:	Room temperature
Solvent:	5mM Ammonium bicarbonate in Water/Acetonitrile (50/50, *v/v*), isocratic
Flow rate:	1000 µL/min
Ion source:	Turbo Spray (ESI)
Polarity:	Positive
Duration:	20 min
Scan type:	MRM (Multiple reaction monitoring) <u>Zoxamide</u> 336.0 →187.0 (quantitation) 338.0 → 189.0 (confirmation)

	RH-127450	
	302.1 →187.0 (quantitation)	
	304.1 → 189.0 (confirmation)	
	Ion spray:	4500V
	Vaporizer temperature:	600°C
MS parameters:	Gas 1:	70 psi
	Gas 2:	70 psi
	Curtain Gas:	50 psi

Equipment method 2 – for the determination of RH-163353

Instrument:	Agilent Infinity II 1260 system equipped with Infinity II 1260 autosampler and Infinity II1260 pump AB Sciex 6500+ QTrap mass spectrometer, electron impact (EI) ion volume Analyst 1.6 LC/MS instrument control and MuliQuant 3.0.2 data acquisition software.
Column:	250 mm x 4.6 mm, 5 µm Phenomenex LUX Cellulose-3
Pre-column:	Security Guard cartridges for LUX Cellulose-3, 4 x 3 mm
Column oven	Room temperature
Solvent:	0.1 % formic acid in Water/Acetonitrile (30/70, *v*/*v*), isocratic
Flow rate:	1000 µL/min
Ion source:	Turbo Spray (ESI)
Polarity:	Negative
Duration:	16 min
Scan type:	MRM (Multiple reaction monitoring) RH-163353 m/z 330 → 159 (quantification) m/z 332 → 161 (confirmation)
MS parameters:	Ion spray: -4500V Vaporizer temperature: 600°C Gas 1: 70 psi Gas 2: 70 psi Curtain gas: 50 psi

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 69: Summary of recovery experiments for zoxamide as sum of the enantiomers - values in percent of applied test item

Zoxamide	LOQ (0.002 mg/kg)	10xLOQ (0.02 mg/kg)
Replicate	**[%]**	**[%]**
a	106.5	79.6
b	104.5	86.4
c	107.5	80.5
d	106.5	102.6
e	102.0	100.9
Mean (n=5)	105.4	90.0
RSD	2.1	12.3
Overall mean (n=10)	97.7	
RSD	11.1	

Table A 70: Summary of recovery experiments for RH-127450 as sum of the enantiomers - values in percent of applied test item

RH-127450	LOQ (0.002 mg/kg)	10xLOQ (0.02 mg/kg)
Replicate	**[%]**	**[%]**
a	110.0	86.0
b	105.0	83.5
c	105.0	80.5
d	105.0	84.0
e	100.0	84.0
Mean (n=5)	105.0	83.6
RSD	3.4	2.4
Overall mean (n=10)	94.3	
RSD	11.6	

Table A 71: Summary of recovery experiments for RH-163353 as sum of the enantiomers - values in percent of applied test item

RH-163353	LOQ (0.002 mg/kg)	10xLOQ (0.02 mg/kg)
Replicate	**[%]**	**[%]**
a	105.0	79.0
b	95.0	81.0
c	100.0	103.5
d	105.0	80.0
e	95.0	84.5
Mean (n=5)	100.0	85.6
RSD	5.0	11.9
Overall mean (n=10)	92.8	
RSD	10.8	

Accuracy and precision / repeatability

The results for accuracy and precision show RSD values of < 20 % for each spiking level and per test item, demonstrating satisfactory accuracy and precision. Recovery values were within 79.6-107.5% for zoxamide, 80.5- 110.0% for RH-127450 and 79.0-105.0% for RH-163353, and thus in the required range of 70-110 %.
Analytes in blank (control) samples were either not detected or showed residues < 30 % of the LOQ.

The mean recoveries of the five LOQ levels were 105.4% with a relative standard deviation of 2.1% (zoxamide), 105% with a relative standard deviation of 3.4% (RH-127450) and 100% with a relative standard deviation of 5% (RH-163353). The mean recoveries of the five 10xLOQ levels were 90.0% with a relative standard deviation of 12.3% (zoxamide), 83.6% with a relative standard deviation of 2.4% (RH-127450) and 85.6% with a relative standard deviation of 11.9% (RH-163353).

Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by a 6-points calibration curve (single injections) using matrix-matched standard solutions over a range of 0.1 to 10 µg/L for zoxamide, RH-127450 and RH-163353. Regression correlation coefficients were > 0.99.

Limit of quantification

The LOQ of this method is 0.002 mg/kg for zoxamide, RH-163353 and RH-127450.

Limit of detection

The LOD of this method is 0.001 mg/kg for zoxamide and metabolites. This is <1% of the applied amount of the test item in the soil degradation study (120 µg/100 g dry soil).

Matrix effects

Matrix effects were evaluated. Both standard solutions and matrix-matched standard solutions were used for quantification.

Specificity

The identity of the analytes was ensured by specific LC-MS/MS with two mass transitions per analyte: RH-127450 (m/z: 302.1 -> 187; m/z: 304.1 -> 189); zoxamide (m/z: 336 -> 187; m/z 338 -> 189); RH-163353 (m/z 330 -> 159; m/z 332 -> 161). Interfering peaks in control samples that eluted at the same retention time as the analytes were either non-detectable or less than 30% of the limit of quantification (LOQ), demonstrating acceptable specificity.

The enantiomeric selection of the analytes has been ensured by enantioselective liquid chromatography coupled with a tandem mass spectrometer detector. Since the reference standards were racemates, the assignment of the analyte's isomers was not possible. Therefore, the separated enantiomers were labelled by their retention times. The isomers of RH-163353 could not be separated.

Stability of sample extracts

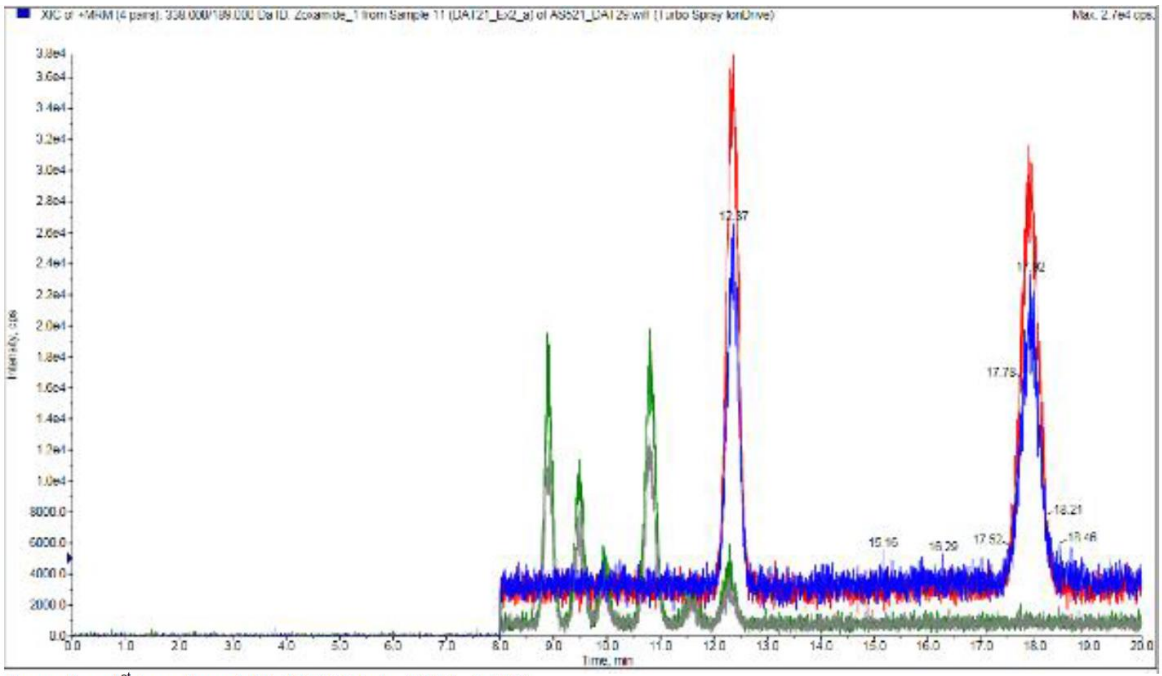
The samples were processed immediately after sampling. Initial extraction procedures were completed within a single working day. Sample extracts were stored frozen at ca -18°C. Initial LC-MS/MS profiles of the extracts were obtained within 8 days of the sample generation. The analysis of RH-163353 was performed 38 days after application (due to a delayed shipment of the analytical standard). Fortified recovery samples processed and stored under the same conditions confirmed the stability of zoxamide in the sample extracts.

Table A 72: Characteristics of the analytical method validation for the determination of zoxamide, RH-127450, RH-163353 (and their enantiomers) in soil

	Zoxamide/ (R/S)-Zoxamide	**RH-127450 (R/S)-RH-127450**	**RH-163353**
Specificity	Mass sprectrum provided. Blank value < 30% of LOQ.	Mass sprectrum provided. Blank value < 30% of LOQ.	Mass sprectrum provided. Blank value < 30% of LOQ.
Calibration (type, number of data points)	Both standard solution and matrix matched standard calibration. 6-point calibration; r^2 >0.99; linear. Examples of calibration data and calibration line equations presented in the report.	Both standard solution and matrix matched standard calibration. 6-point calibration; r^2 >0.99; linear. Examples of calibration data and calibration line equations presented in the report.	Both standard solution and matrix matched standard calibration. 6-point calibration; r^2 >0.99; linear. Examples of calibration data and calibration line equations presented in the report.
Calibration range	0.1 to 10 µg/L	0.1 to 10 µg/L	0.1 to 10 µg/L
Assessment of matrix effects is presented	yes	Yes	yes

	Zoxamide/ (R/S)-Zoxamide	**RH-127450 (R/S)-RH-127450**	**RH-163353**
Specificity	Mass sprectrum provided. Blank value < 30% of LOQ.	Mass sprectrum provided. Blank value < 30% of LOQ.	Mass sprectrum provided. Blank value < 30% of LOQ.
Limit of determination/quantification	LOQ: 0.002 mg/kg LOD: 0.001 mg/kg	LOQ: 0.002 mg/kg LOD: 0.001 mg/kg	LOQ: 0.002 mg/kg LOD: 0.001 mg/kg

The following figures show chromatograms.



Green line: 1st transition of RH-127450 (m/z: 302.1 → 187)
Grey line: 2nd transition of RH-127450 (m/z: 304.1 → 189)
Red line: 1st transition of Zoxamide (m/z: 336 → 187)
Blue line: 2nd transition of Zoxamide (m/z 338 → 189)

Green line: 1st transition of RH-127450 (m/z: 302.1 → 187)
Grey line: 2nd transition of RH-127450 (m/z: 304.1 → 189)
Red line: 1st transition of Zoxamide (m/z: 336 → 187)
Blue line: 2nd transition of Zoxamide (m/z 338 → 189)

Figure A 34: LC-MS/MS chromatogram of zoxamide and RH-127450 in soil extract (above) and in fortified control sample at a concentration of 1.2 mg/kg (below)

Conclusion

The analytical method has been sufficiently validated according to SANCO/825/00 and SANCO/3029/99 for the monitoring of zoxamide, RH-127450 and RH-163353 in Mußbach soil at an LOQ of 0.002 mg/kg.

The method is capable for the determination of (R)- and (S)-enantiomers of zoxamide and its metabolite RH-127450. The enantioselective separation of the metabolite RH-163353 could not be achieved.

(Kercher S. 2017)

A 2.1.1.7.2 Analytical method 2 – determination of zoxamide in soil based on Jooß S. 2013 (see RAR zoxamide, 2017)

The following studies applied the post-registration HPLC-MS/MS method of Jooß (2013), which has been evaluated during AIR of zoxamide (see RAR of zoxamide, 2017).

A 2.1.1.7.2.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/52**
Report	Friedrich, S., 2020: Effects of Zoxium 240 SC on the reproduction of the earthworm *Eisenia andrei* in artificial soil 5% peat Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 17 48 TEC 0009, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
Acceptability:	Yes

Materials and methods

A highly specific HPLC-MS/MS method for the determination of zoxamide in soil has been validated according to SANCO/3029/99 rev. 4.

The active substance zoxamide was analysed in soil specimens by a method developed by Jooß (2013), using extraction with acetonitrile and separation by reverse-phase high-pressure liquid chromatography (HPLC) with tandem mass spectroscopic (MS/MS). Matrix-matched external standards were used. The method of Jooß (2013) has been adapted to the expected concentration range of this study.

5 g (± 0.05 g) soil sample were weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of the acetonitrile phase were transferred to autosampler vials and diluted.

The analytes were determined after extraction with two mass transitions (zoxamide: m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Aglient 1200/64100 system with a triple quadrupole mass spectrometric detector
Column:	ACE Excel 3µm C18-AR 100*2.1 mm
Mobile phase:	A: Water containing 1 mL/L formic acid and 5 mmol/L ammonium formate B: Methanol containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00		Stop

Flow rate:	0.4 mL/min
Run time:	7.00 min (3.0 min post-run equilibration)
Ionisation:	ESI (electrospray ionisation) positive
Ion mode	Zoxamide: m/z 336 → m/z 159 (quantifier ion) m/z 336 → m/z 187 (qualifier ion)

Results and discussions

Table A 73: Summary of recoevery experiments

Validation	Replicates	Nominal conc. (mg/kg dry weight)	Nominal conc. (mg/kg moist soil)	Main analysed conc. (mg/kg)	Mean recovery (%)	RSD (%)
Low	5	0.063	0.051	0.050	99	3.8
Medium	5	0.632	0.506	0.509	101	2.2
High	5	29.09	23.28	23.82	102	2.7

Accuracy and precision / repeatabiltiy

The results for accuracy and precision showed RSD values < 10 % for each spiking level and overall. Recoveries were in the expected range of 70-110%.
Untreated samples showed residues < LOD.
Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was demonstrated for matrix-matched calibration curves (8 concentration levels) of zoxamide in the range of 0.80 – 84 µg/L (corresponding to 0.05 – 5.25 mg /kg zoxamide in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients r^2 all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 0.051 mg/kg zoxamide in wet soil specimens, equivalent to 0.063 mg/kg zoxamide in dry weight soil.

Matrix effects

Matrix effects were compensated by matrix-matched calibration standards

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as zoxamide, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability

Sample extracts and solvent standards were stored for max. two days in the refrigerator or the cooled autosampler (at 4-8°C). A stability of 8 days for zoxamide in sample extracts and for solvent standard solutions was demonstrated in BioChem study no. 18 35 CRX 0023 by Thomas (2020).

Frozen soil samples (≤-18°C) were stored during the course of this study for at maximum 425 days. Storage stability of zoxamide in deep frozen soil samples has been demonstrated for a period of 633 days in BioChem study no. 18 35 CRX 0023 by Thomas (2020).

Table A 74: Characteristics of the analytical method validation for the determination of zoxamide in artificial soil

	Zoxamide
Specificity	Mass sprectrum provided. HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	8-point calibration with matrix-matched standard. The calibration was linear. Calibration curve equation: $y = 192.895 x + 34.631$, $r^2=0.99944$
Calibration range	0.80 – 84 µg/L in analytical samples (corresponding to 0.05 – 5.25 mg /kg zoxamide in dry soil)
Assessment of matrix effects is presented	No (matrix-matched standards).
Limit of quantification	0.051 mg/kg zoxamide in moist soil (as received), equivalent to 0.063 mg/kg zoxamide in dry soil and 1.0 µg./L in the analytical sample.

The following figure shows a representative chromatogram.

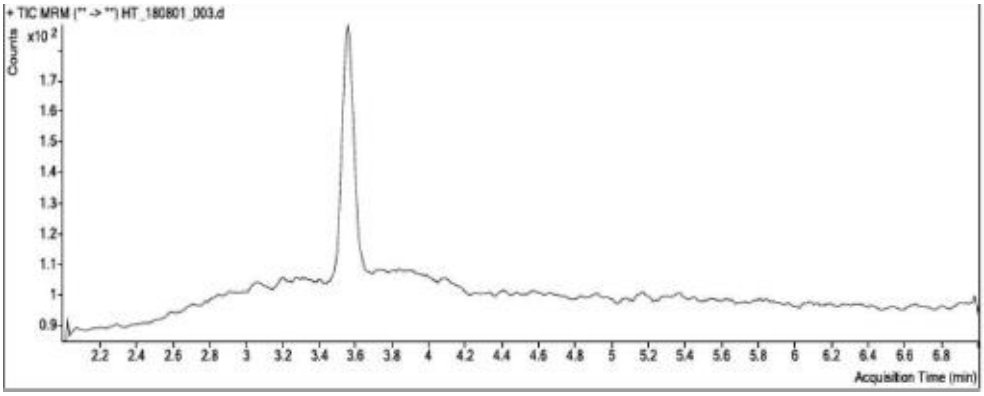


Figure A 35: Chromatogram of the lowest zoxamide standard

Conclusion

A highly specific HPLC-MS/MS method for the determination of zoxamide in soil has been validated according to SANCO/3029/99 rev. 4.

(Friedrich S. 2020)

A 2.1.1.7.2.2 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/53**
Report	Schulz, L., 2020: Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK, BioChem agrar, Germany, Report No.18 48 FEW 0001, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the analytical verification of the soil concentrations of zoxamide after application of the test item Zoxium 240 SC (containing nominally 240 g/L zoxamide).

The determination of zoxamide (sum of enantiomers) was conducted by a HPLC-MS/MS method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 (Jooß S., 2013). It was adapted to the equipment available at BioChem agrar. Changes to the original method are reported and laboratory validation data according to SANCO/3029/99 were compiled prior to the test item measurements.

5 g soil were weighed (to ±0.05 g) into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 200 µL of the acetonitrile phase were pipetted into autosampler vials and diluted with 790 µL water and 10 µL formic acid. After extraction, the analyte was determined monitoring two mass transitions (m/z 336 -> 187 and 336 -> 159), one for quantification and one for qualification, respectively.

Equipment

HPLC parameters for chiral separation of zoxamide stereoisomers:

Instrument:	Shimadzu LC-20ADXRsystem with an LCMS-8040mass spectrometric detector
Column:	ACE Excel 3 µm C18-AR 100 * 2.1 mm

Mobile phase: A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate
B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Zoxamide:
m/z 336 → m/z 187 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 75: Validation results

Validation level	Replicates	Nominal concentration mg/kg d.w.	Mean Analysed concentrations mg/kg d.w.	Mean recovery [%]	RSD [%]
Low	5	0.0563	0.0577	102	5.9
High	5	0.563	0.614	109	4.4

Accuracy and precision / repeatability

The accuracies, reported as mean recovery and precision / repeatability as relative standard deviation are shown in table above. Mean recoveries for each level are in the range 70-110%, the RSD is < 20% per level. The results fulfill the criteria of SANCO/3029/99 rev. 4.

Linearity

Linearity was demonstrated for matrix-matched calibrations (6 concentration levels) in the range of 0.78 to 12.0 µg/L, with a correlation coefficient (r) greater than 0.99. This is equivalent to 0.044 - 0.68 mg/kg zoxamide in dry soil.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 0.056 mg/kg zoxamide, per soil dry weight.

Matrix effects

Matrix-matched calibration was used to compensate possible matrix effects. Nevertheless no significant matrix effect was found, comparing standards prepared in matrix and in solvent.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two mass transitions per analyte for quantification and confirmation. No interfering peaks and no blank values above 30% of LOQ were detected.

Storage stability of sample extracts and solvent standards

Sample extracts were stable for 8 days at 4-8°C.

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 639 days. The freezer storage stability of zoxamide residues in soil samples was confirmed for 643 days (21 months). For this, freshly spiked soil samples were analysed concurrently to the stored samples in the freezer.

Table A 76: Characteristics of the analytical method used for determination of zoxamide in field soil

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point calibration with external standard. Individual calibration data presented in the report The calibration was linear over the whole calibration range. Calibration curve equation: $y = 12540.6\ x - 341.9$, $r^2 = 0.9996$
Calibration range	0.78 to 12.04 µg/L in soil extracts; Equivalent to 0.044 to 0.684 mg/kg zoxamide in dry soil
Assessment of matrix effects	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of quantification (LOQ)	0.056 mg/kg zoxamide per soil dry weight

The following figure shows a representative chromatogram.

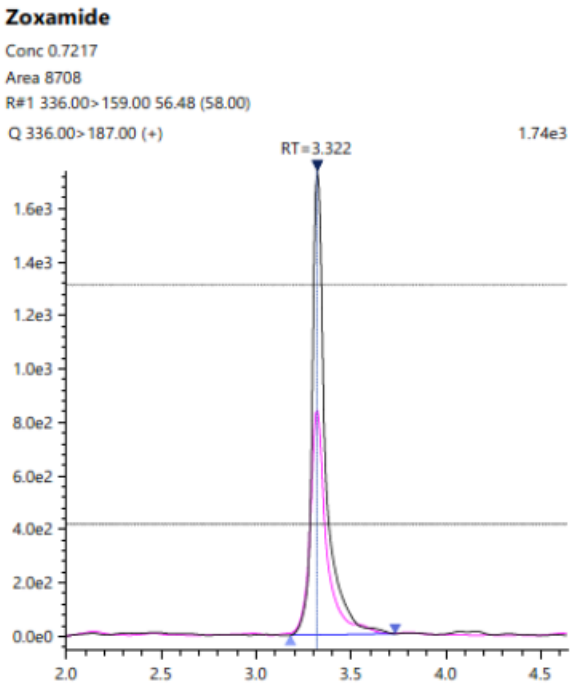


Figure A 36: Chromatogram of zoxamide of the lowest matrix standard

Conclusion

The analytical method is applicable for the determination of zoxamide in soil. It fulfills the criteria of SANCO/3029/99 rev. 4.

(Schulz L. 2020)

A 2.1.1.7.2.3 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/54**
Report	Parsons, Ch., 2020: Zoxium 240 SC– A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, BioChem agrar, Germany, Report No. GOW-17-13, GLP, Not Published
Guideline(s):	OECD 232, 2016
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial standard soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of BioChem agrar was used. This has no influence on the integrity of the study results. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to determine the zoxamide concentrations in soil specimens. The determination of zoxamide (sum) was conducted by a method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 by PTRL. The method was provided by the Sponsor (Jooß, 2013). It was adapted to the equipment available at BioChem agrar. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide. Actually the LOQ as determined in the original method validation (Jooß, 2013) is 0.5 mg/kg zoxamide.

5 g soil (weighed to ±0.05 g) was weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 10 µL of the acetonitrile phase were transferred to autosampler vials and diluted with 990 µL 0.125% formic acid in water (v/v). The analyte was determined after extraction with two mass transitions (m/z 336 -> 187 and 336 -> 159), one for quantification and one for qualification, respectively.

Equipment

LC-MS System: Shimadzu LC20 HPLC system, Shimadzu 8040 LC-MS/MS detector.
Column: ACE Excel 3µm C18-AR 100 * 2.1 mm
Mobile phase: A: water containing 1mL/L formic acid
B: acetonitrile containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0	50	50
4	20	80
7	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI positive
Detection: m/z 336 → m/z 159 (quantifier ion)
m/z 336 → m/z 187 (qualifier ion)

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 77: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1000	n.d.		n.d.		n.d.	
A, zoxamide	168.6	1000	188.8	112	167.8	100	165.4	98

Table A 78: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1250	n.d.		n.d.		n.d.	
A, zoxamide	210.8	1250	236.0	112	209.8	100	206.8	98

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the table below.

Table A 79: Re-Validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	1000	19.03	18.50	97	3.4
High	5	1000	190.3	188.7	99	3.0
Concentration in dry soil						

Low	5	1250	23.79	23.13	97	3.4
High	5	1250	237.9	235.9	99	3.0

Repeatability data was generated from five samples of test media fortified at the LOQ and five samples fortified at the highest nominal concentration of the test samples. The relative standard deviations (RSD) obtained at each fortification level were within the guideline requirements.

Linearity

The calibration was slightly non-linear, a quadratic fit with 1/c weighting was used in the range of 15.1 to 229 µg/L. This covers the range from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with a correlation coefficient ® greater than 0.99. Accuracy was maintained by the measurement of recalibration standards before and after sample measurements.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However, the LOQ of the original method (Jooß, 2013) is 0.05 mg/kg zoxamide.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Stability of sample extracts

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Table A 80: Characteristics for the confirmatory method used for validation of zoxamide residues in Folsomia

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data presented in section 7.1. Calibration curve equations: $y = -1.923\ x^2 + 6565.17\ x + 4540.73$, r^2=0.9999995
Calibration range	15 to 229 µg/L in analytical samples (15 to 229 mg/kg in soil)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30%

	Zoxamide
	of the lowest validation samples).
Limit of determination/quantification	19 mg/kg a.i. in moist soil (as received), corresponding to 24 mg/kg a.i. in dry soil and 19 µg/L a.i. in the analytical sample.

The following figure shows typical chromatogram.

17X24-25-Cal 1

Sample ID:
Date acquired: 15.11.2018 18:23:50
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_04.lcd
Vial: 2 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	2	Standard	15.11.2018 18:23:50	3.362	101195	32142	15.1006	99.95	56.28

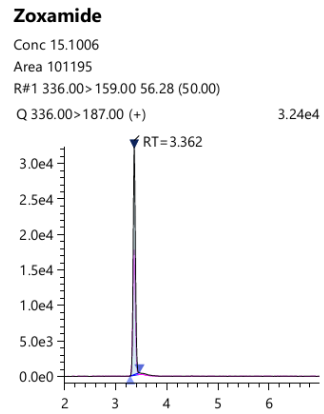


Figure A 37: Chromatogram of the lowest standard

17X24-25-Cal 5

Sample ID:
Date acquired: 15.11.2018 19:48:15
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_12.lcd
Vial: 6 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	6	Standard	15.11.2018 19:48:15	3.361	1383807	430928	229.7445	100.36	56.07

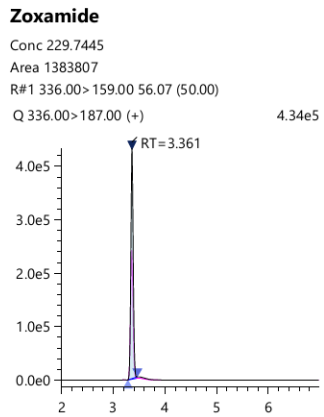


Figure A 38: Chromatogram of highest standard

Conclusion

The nominal initial concentration in the fresh soil specimen could be confirmed – the recovery was greater than 80% (112%). The zoxamide concentrations stayed within the range of 98 – 112% of nominal throughout the whole study period.

(Parsons Ch. 2020)

A 2.1.1.7.2.4 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/55**
Report	Parsons, Ch., 2020: Zoxium 240 SC – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, BioChem agrar, Germany, Report No. GOW-17-14, GLP, Not Published
Guideline(s):	OECD 226, 2016
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial standard soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of Bio-Chem agrar was used. This has no influence on the integrity of the study results. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredient zoxamide was analysed by a validated method (Jooß, 2013). Actually, the LOQ of the original method is 0.05 mg/kg soil d.w. However, the method was adapted to the expected concentration range of this study and to the equipment available. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% $CaCO_3$, wetted to 20% water content) spiked with test item at concentrations of 19 mg/kg a.i. in moist soil (24 mg/kg a.i. or 113 mg/kg test item in dry soil) and at 190 mg/kg a.i. in moist soil (238 mg/kg a.i. or 1129 mg/kg test item in dry soil) with 5 replicates per fortification level. This amounts to a working range of approximately 25 - 110% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

5 g soil (weighed to ±0.05 g) was weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 10 µL of the acetonitrile phase were transferred to autosampler vials and diluted with 990 µL 0.125% formic acid in water (v/v). The analyte was determined after extraction with two mass transitions (m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

LC-MS System:	Shimadzu LC20 HPLC system, Shimadzu 8040 LC-MS/MS detector.
Column:	ACE Excel 3µm C18-AR 100 * 2.1 mm

Mobile phase: A: water containing 1mL/L formic acid
B: acetonitrile containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI positive
Detection: m/z 336 → m/z 159 (quantifier ion)
m/z 336 → m/z 187 (qualifier ion)

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 81: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1000	n.d.		n.d.		n.d.	
A, zoxamide	168.6	1000	171.4	102	180.2	107	184.6	109

Table A 82: Analysis results in dry soil

Treatment group	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	n.d.		n.d.		n.d.	
A, zoxamide	210.8	214.3	102	225.3	107	230.8	109

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the table below.

Table A 83: Re-Validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	1000	19.03	18.50	97	3.4
High	5	1000	190.3	188.7	99	3.0
Concentration in dry soil						
Low	5	1250	23.79	23.13	97	3.4
High	5	1250	237.9	235.9	99	3.0

Repeatability data was generated from five samples of test media fortified at the LOQ and five samples fortified at the highest nominal concentration of the test samples. The relative standard deviations (RSD) obtained at each fortification level were within the guideline requirements.

Linearity

The calibration was slightly non-linear, a quadratic fit with 1/c weighting was used in the range of 15.1 to 229 µg/L. This covers the range from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with a correlation coefficient R greater than 0.99. Accuracy was maintained by the measurement of recalibration standards before and after sample measurements.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However, the LOQ of the original method (Jooß, 2013) is 0.05 mg/kg zoxamide.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Stability of sample extracts

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Table A 84: Characteristics for the confirmatory method used for validation of zoxamide residues in Folsomia

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data presented in section 7.1. Calibration curve equations: $y = -1.923\ x^2 + 6565.17\ x + 4540.73$, r^2=0.9999995
Calibration range	15 to 229 µg/L in analytical samples (15 to 229 mg/kg in soil)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/quantification	19 mg/kg a.i. in moist soil (as received), corresponding to 24 mg/kg a.i. in dry soil and 19 µg/L a.i. in the analytical sample.

Following figure show chromatograms.

17X24-25-Cal 1

Sample ID:
Date acquired: 15.11.2018 18:23:50
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_04.lcd
Vial: 2 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	2	Standard	15.11.2018 18:23:50	3.362	101195	32142	15.1006	99.95	56.28

Zoxamide

Conc 15.1006
Area 101195
R#1 336.00>159.00 56.28 (50.00)
Q 336.00>187.00 (+) 3.24e4

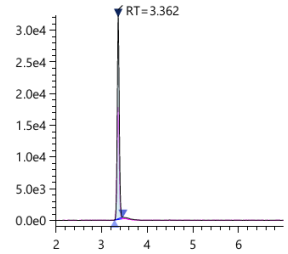


Figure A 39: Chromatogram of the lowest standard

17X24-25-Cal 5

Sample ID:
Date acquired: 15.11.2018 19:48:15
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_12.lcd
Vial: 6 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	6	Standard	15.11.2018 19:48:15	3.361	1383807	430928	229.7445	100.36	56.07

Zoxamide

Conc 229.7445
Area 1383807
R#1 336.00>159.00 56.07 (50.00)
Q 336.00>187.00 (+) 4.34e5

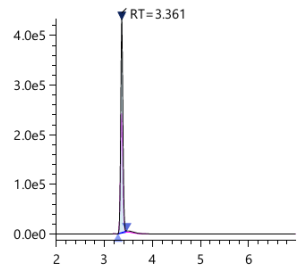


Figure A 40: Chromatogram of highest standard

Conclusion

The nominal initial concentration in the fresh soil specimen could be confirmed – the recovery was greater than 80% (102%). The zoxamide concentrations stayed within the range of 102 – 109% of nominal throughout the whole study period.

(Parsons Ch. 2020)

A 2.1.1.7.3 Analytical method 3 – determination of zoxamide and cymoxanil in soil (the

zoxamide method is based on Jooß S. 2013; see RAR zoxamide, 2017)

The following studies applied the post-registration HPLC-MS/MS method of Jooß (2013) for the determination of zoxamide in soil, which has been evaluated during AIR of zoxamide (see RAR of zoxamide, 2017).

A 2.1.1.7.3.1 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are in the required range.

Reference:	**KCP 5.1/56**
Report	Thomas, H., 2020: Validation of a HPLC-MS-MS method for the determination of cymoxanil and zoxamide in soil Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A. BioChem agrar, Germany, Report No.18 35 CRX 0033, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The objective of the study was the development and validation of an analytical method for the determination of cymoxanil and zoxamide, the active ingredients in Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823; trade name e.g. REBOOT), to confirm the applied test item concentrations in soil specimens of ecotoxicological studies.

The method was developed based on Jooß (2013) and Melkebeke (2000), using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC), and tandem mass (MS/MS) determination of the analytes with matrix-matched external standards. It is regarded as highly specific for the determination of the analytes with two mass transitions (cymoxanil: m/z 199.1 → 128.1 and 199.1 → 111.0, zoxamide: m/z 336.0 → 187.0 and 336.0 → 159.0), for quantification and/or qualification, respectively. The limit of quantification (LOQ) was chosen at 0.0125 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. The method was fully validated at the LOQ (0.0125 mg/kg soil d.w.), 10x LOQ (0.125 mg/kg soil d.w.) and 100x LOQ (1.25 mg/kg soil d.w.) according to guidance document SANCO/3029/99 rev. 4 (11/07/2000) using two different soil types, a sandy loamy silt from Saxony and an artificial soil substrate with 10 % peat.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugated. Aliquots of the acetonitrile phase were diluted with a solution of 50:50 (v/v) methanol/water containing 0.1% formic acid to the validated concentration range and analysed.

An external calibration (2.0 to 30 µg/L for both cymoxanil and zoxamide) with the reference items was performed. The calibration spanned from less than 80% of the lowest validation concentration to more than 120% of the highest validation concentration (taking into account the sample preparation and dilution).

Equipment

Instrument: Triple Qudrupole Shimadzu LCMS-8040
Column: ACE Excel 3 C18-AR, 100 * 2.1 mm
Mobile phase: A: water containing 0.1 % formic acid and 5 mmol/L ammonium formate
B: methanol containing 0.1 % formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate: 0.4 mL/min
Column temp.: 40°C
Injection volume: 1 µL
Retention time: Cymoxanil: approx. 3.95 min
Zoxamide: approx. 6.05 min
Run time: 9.0 min
Ionisation: ESI (electrospray ionisation) positive
Ion mode Cymoxanil:
m/z 199.1 → m/z 128.1 (quantifier ion)
m/z 199.1 → m/z 111.0 (qualifier ion)
Zoxamide:
m/z 336 → m/z 187 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 85: Recovery results from method validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg a.i. in moist soil)	n	Mean recovery (%)	RSD (%)	Comments
Soil	Cymoxanil	0.01	2 x 5	91	9.5	Pooled RSD from two soils
Soil	Cymoxanil	0.10	2 x 5	94	2.0	Pooled RSD from two soils
Soil	Cymoxanil	1.01	2 x 5	92	1.3	Pooled RSD from two soils
Soil	Zoxamide	0.01	2 x 5	98	8.6	Pooled RSD from two soils
Soil	Zoxamide	0.10	2 x 5	96	0.7	Pooled RSD from two soils
Soil	Zoxamide	1.01	2 x 5	91	0.9	Pooled RSD from two soils

RSD = relative standard deviation
n = sample size

Accuracy and precision / repeatability

The results were in the range of 70 – 110 % with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for both analytes over the whole calibration range. The calibration ranges were between 2.0 to 30 µg/L with 6 concentration levels for each matrix-matched calibration curve (corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight).This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r^2) all greater than 0.999, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 0.01 mg/kg a.i. in moist soil, equivalent to 0.0125 mg/kg dry weight.

Limit of detection

The limit of detection (LOD) is estimated at 0.003 mg/kg a.i. for cymoxanil and zoxamide (0.004 mg/kg dry weight).

Matrix effects

Matrix effects were evaluated during non-GLP method development. Standards in soil extract matrix showed a reduction in peak area of about 27% for cymoxanil and about 10% for zoxamide compared to standards in solvent. Matrix-matched standard solutions were therefore used for the quantification of cymoxanil and zoxamide in the sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as zoxamide or cymoxanil were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for at max. 8 days in the cooled autosampler. Therefore, recovery experiments were performed and demonstrated that the analytes in soil extracts were stable for at least 8 days when stored at temperatures of 4-8 °C (in the refrigerator or the cooled autosampler).

Storage stability of frozen samples

The stability of frozen samples is investigated in a separate study.

Table A 86: Characteristics of the analytical method validation for the determination of zoxamide and cymoxanil in artificial soil

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point calibration with external matrix-matched standard The calibration was linear. Calibration curve equation: $y = 1602.523\ x + 45.27193$, $r^2 = 0.99990$	6-point calibration with external matrix-matched standard The calibrations was linear. Calibration curve equation: $y = 63612.26\ x + 27042.29$, $r^2 = 0.99997$
Calibration range	2.0 to 30 µg/L in soil extracts, corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight	2.0 to 30 µg/L in soil extracts, corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had no peaks >30% of the lowest validation samples.	Recoveries of validation samples within 70-110%. Validation blank samples had no peaks >30% of the lowest validation samples.
Limit of determination/ quantification	LOQ: 0.01 mg/kg moist soil; 0.0125 mg/kg soil dry weight LOD: 0.003 mg/kg moist soil, 0.004 mg/kg soil dry weight	LOQ: 0.01 mg/kg moist soil; 0.0125 mg/kg soil dry weight LOD: 0.003 mg/kg moist soil, 0.004 mg/kg soil dry weight

The following figure shows a representative chromatogram.

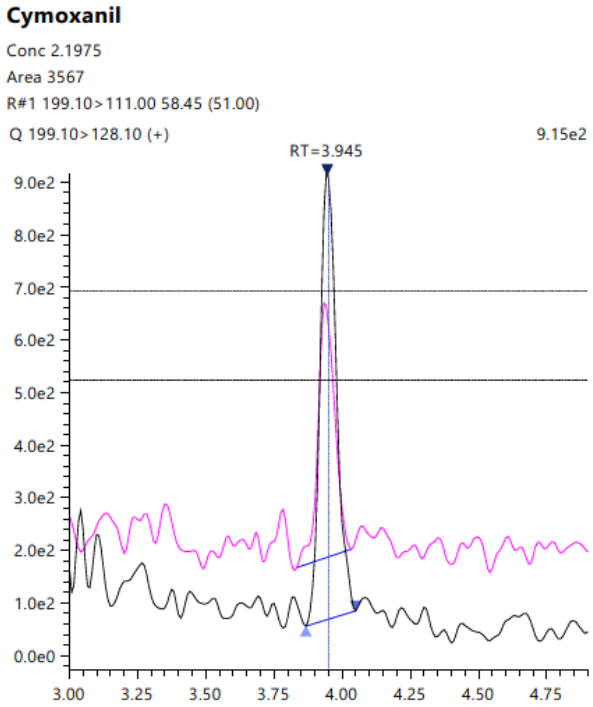


Figure A 41: Chromatogram of cymoxanil

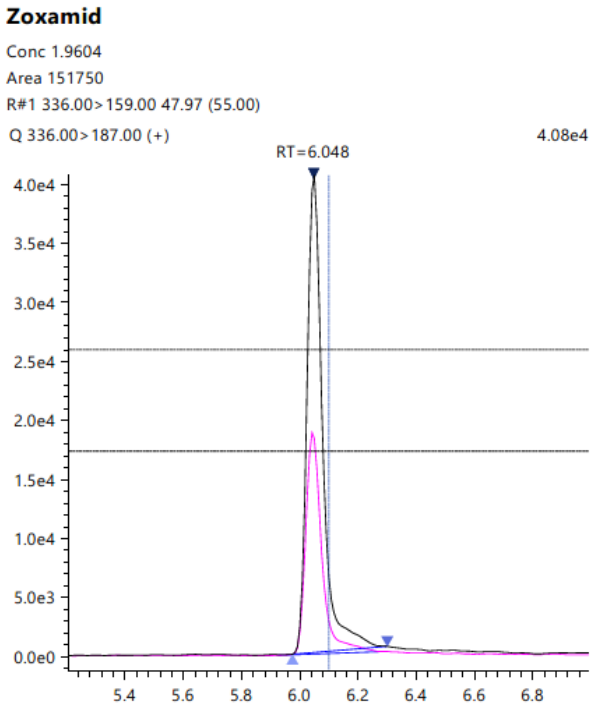


Figure A 42: Chromatogram of zoxamide

Conclusion

A HLPC method with MS/MS detection for the determination of cymoxanil and zoxamide in soil was fully validated according to SANCO/3029/99 rev.4. With this method, cymoxanil and zoxamide can be reliably determined in moist soil from 0.01 to 1.0 mg/kg (0.0125 to 1.25 mg/kg dry weight.

(Thomas H. 2020)

A 2.1.1.7.3.2 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/57**
Report	Friedrich, S., 2020: Effects of Cymoxanil 33 % + Zoxamide 33 % WG on the reproduction of the earthworm *Eisenia andrei* in artificial soil Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No.17 48 TEC 0008, 18 35 CRX 0029, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (report no. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (report no. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (10 % peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with 5 replicates per fortification level at test item at concentrations of approximately 50% of the lowest test concentration (0.55 mg a.i./kg moist soil or 0.74 mg a.i./kg soil d.w.) and approximately 120% of the highest test concentration (38 mg a.i./kg moist soil or 51 mg a.i./kg soil d.w.). Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed.

The analytes were determined in the diluted extraction samples with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 →111), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Shimadzu LC-20 system with an 8040 triple quadrupole mass spectrometric detector
Column:	ACE Excel 3 C18-AR, 100 * 2.1 mm

Mobile phase: A: water containing 0.1 % formic acid and 5 mmol/L ammonium formate
B: methanol containing 0.1 % formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate: 0.4 mL/min
Column temp.: 40°C
Injection volume: 1 µL
Retention time: Cymoxanil: 3.3 - 3.4 min
Zoxamide: 5.4 - 5.5 min
Run time: 9.0 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Cymoxanil:
m/z 199.1 → m/z 128.1 (quantifier ion)
m/z 199.1 → m/z 111.0 (qualifier ion)
Zoxamide:
m/z 336 → m/z 186.95 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 87: Analysis results in the soil specimens (based on soil dry weight)

Treatment group	Nominal concentration [mg/kg dw]	Day 0		Day 28		Day 56	
		analysed concentration [mg/kg dw]	recovery [%]	analysed concentration [mg/kg dw]	recovery [%]	analysed concentration [mg/kg dw]	recovery [%]
Cymoxanil							
1	0.000	n.d.		n.d.		n.d.	
2	1.509	1.312	87%	0.343 [x]	23	0.129 [x]	8.5
3	2.716	2.449	90%	0.606	22	0.196 [x]	7.2
4	4.889	4.241	87%	1.199	25	0.358 [x]	7.3
5	8.801	7.770	88%	2.282	26	0.759	8.6
6	15.84	14.82	94%	3.681	23	1.076	6.8
7	28.52	26.57	93%	7.585	27	2.062	7.2
8	51.33	46.05	90%	17.960	35	7.487	15
Zoxamide							
1	0.000	n.d.		n.d.		n.d.	

2	1.482	1.200	81	0.394[x]	27	0.143 [x]	10
3	2.667	2.341	88	0.847	32	0.306 [x]	11
4	4.801	4.196	87	1.641	34	0.628 [x]	13
5	8.642	7.673	89	2.857	33	1.289	15
6	15.56	14.61	94	5.298	34	2.184	14
7	28.00	27.52	88	9.839	35	4.007	14
8	50.40	44.98	89	20.844	41	8.874	18

mg/kg dw = mg/kg corrected to soil dry weight
n.d. = not detected
[x] = result below the LOQ validated in this phase

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation is shown in the table below.

Table A 88: Re-validation results

Validation	Replicates	Sample preparation factor [mL/g]	Nominal conc. [mg/kg]	Mean analysed conc. [mg/kg]	Mean recovery [%]	RSD [%]
Cymoxanil (concentration in moist soil)						
Low	5	40	0.559	0.468	84	2.9
High	5	200	38.24	33.82	88	1.0
Zoxamide (concentration in moist soil)						
Low	5	40	0.549	0.485	88	2.4
High	5	200	37.55	34.78	93	1.6
Cymoxanil (concentration in dry soil)						
Low	5	40	0.754	0.632	84	2.9
High	5	200	51.63	45.66	88	1.0
Zoxamide (concentration in dry soil)						
Low	5	40	0.741	0.655	88	2.4
High	5	200	50.69	46.96	93	1.6

Linearity

Linearity was demonstrated for solvent calibration curves (6 concentration levels) of zoxamide in the range of 11.00 – 224.55 µg/L (corresponding to 0.44 – 45 mg/kg moist soil and 0.59 – 61 mg/kg dry soil) and cymoxanil in the range of 11.27 – 230.01 µg/L (corresponding to 0.45 – 46 mg/kg moist soil and 0.61 – 62 mg/kg dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 0.56 mg/kg cymoxanil and 0.55 mg/kg zoxamide in moist soil

specimens, equivalent to 0.75 mg/kg cymoxanil and 0.74 mg/kg zoxamide in dry weight soil and 14 µg/L cymoxanil and 14 µg/L zoxamide in diluted extracts. However, the LOQ of the original method (report no. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Limit of detection

The limit of detection (LOD) was defined in the context of this phase of the study as the lowest calibration level, i.e. 11.00 µg/L of zoxamide and 11.27 µg/L of cymoxanil, equivalent to 0.45 mg/kg cymoxanil and 0.44 mg/kg zoxamide in moist soil specimens, equivalent to 0.61 mg/kg cymoxanil and 0.60 mg/kg zoxamide in dry weight soil

Matrix effects

Matrix effects were assessed by evaluating the recovery results from spiked and unspiked control samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, the analytes showed characteristic retention times and interfering peaks in control samples, which eluted at the same retention time as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for at max. 23 hours in the refrigerator. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated

Table A 89: Characteristics of the analytical method used for determination of zoxamide and cymoxanil in artificial soil

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point calibration with external standard. Individual calibration data presented in section 7.1 of the study report. The calibration was linear. Calibration curve equation: $y = 9473.322 x + 21465.57$, $r^2=0.99945$	6-point calibration with external standard. Individual calibration data presented in section 7.1 of the study report. The calibration was linear. Calibration curve equation: $y = 9154.537 x – 5734.254$, $r^2=0.99996$
Calibration range	11.00 to 224.55 µg/L in the diluted soil extract (0.44 to 45 mg a.s./kg moist soil and 0.59	11.27 to 230.01 µg/L in the diluted soil extract (0.45 to 46 mg a.s./kg moist soil and 0.61

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
	to 61 mg a.s./kg dry soil) ~~(xx to yy mg a.s./kg moist soil and xx to yy mg a.s./kg dry soil)~~	to 62 mg a.s./kg dry soil) ~~(xx to yy mg a.s./kg moist soil and xx to yy mg a.s./kg dry soil)~~
Assessment of matrix effects	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of quantification (LOQ)	0.549 mg a.s./kg moist soil (as received), corresponodig to 0.741 mg a.s./kg dry soil and 13.71 µg a.s./L in the analytical sample.	0.559 mg a.s./kg moist soil (as received), corresponodig to 0.754 mg a.s./kg dry soil and 13.97 µg a.s./L in the analytical sample.

The following figure shows a representative chromatogram.

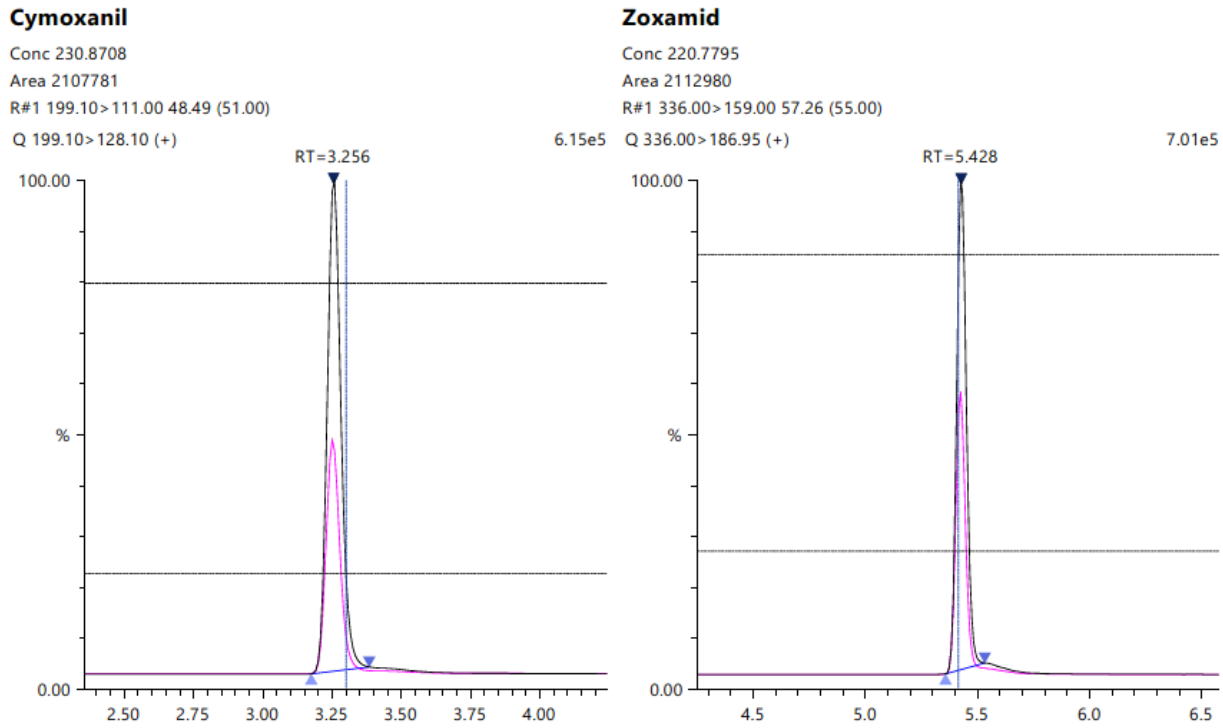


Figure A 43: Chromatograms of the highest calibration standard in 50% methanol containing 0.1% formic acid

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (87-94% for cymoxanil and 81-94% for zoxamide). After 28 days, the cymoxanil concentrations had declined to 22 – 35% of nominal, the zoxamide concentrations to 27 – 41% of nominal. After 56 days, the cymoxanil concentrations had declined to 7 – 15% of nominal, the zoxamide concentrations to 10 – 18% of nominal.

(Friedrich S. 2020)

A 2.1.1.7.3.3 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/58**
Report	Schulz, L., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on earthworms under field conditions Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No.19 48 FEW 0003, 19 35 CRX 0030, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	Volume of acetonitrile used for extraction: 10 mL acetonitrile instead of 20 mL were used for extraction of the soil samples. This has no impact on the outcome of the analytical phase.
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the analytical verification of the concentrations of zoxamide and cymoxanil in field soil and spray targets. The determination was conducted by a validated method, and was re-validated in this study according to SANCO/3029/99 rev.4. In addition, the isomer ratio of (R)- and (S)-zoxamide was determined in selected samples to confirm the chiral stability of zoxamide (racemate).

10 g soil were wetted with 2 mL water and extracted with 10 mL acetonitrile and 0.2 mL acetic acid for 15 minutes on a vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 (w/w) mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were analysed.

Two mass transitions of each analyte were used for detection (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 → 111), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Shimadzu LC-20ADXRsystem with an LCMS-8040mass spectrometric detector
Column:	ACE Excel 3 C18-AR, 100 * 2.1 mm

Mobile phase: A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate
B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate: 0.4 mL/min
Column temp.: 40°C
Run time: 9.0 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Cymoxanil:
m/z 199.1 → m/z 128.1 (quantifier ion)
m/z 199.1 → m/z 111.0 (qualifier ion)
Zoxamide:
m/z 336 → m/z 186.95 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

HPLC parameters for chiral separation of zoxamide stereoisomers

Instrument: Shimadzu LC-20ADXRsystem with an LCMS-8040mass spectrometric detector
Column: Phenomenex Lux Cellulose-3, 150 * 2 mm, 3 µm
Mobile phase: A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate
B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	40	60
8.00	20	80
8.01	0	100
10.00	0	100
10.01	40	60

Flow rate: 0.4 mL/min
Run time: 12.5 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Zoxamide:
m/z 336 → m/z 187 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 90: Re-validation results for cymoxanil

Validation level	Nominal concentration mg/kg d.w.	Analysed concentrations mg/kg d.w.	Mean analysed concentration mg/kg d.w.	Mean recovery [%]	RSD [%]
Low (quantifier transition)	0.0118	0.0093, 0.0093, 0.0095, 0.0098, 0.0095	0.0095	80	2.2

High (quantifier transition)	2.350	2.091, 2.035, 2.100, 2.064, 1.947	2.047	87	3.0
Low (qualifier transition)	0.0118	0.0085, 0.0080, 0.0105, 0.0089, 0.0094	0.0090	76	10.5
High (qualifier transition)	2.350	2.115, 2.043, 2.103, 2.105, 1.967	2.067	88	3.0

Table A 91: Re-validation results for Zoxamide

Validation level	Nominal concentration mg/kg d.w.	Analysed concentrations mg/kg d.w.	Mean analysed concentration mg/kg d.w.	Mean recovery [%]	RSD [%]
Low (quantifier transition)	0.0118	0.0086, 0.0086, 0.0082, 0.0084, 0.0085	0.0084	71	1.7
High (quantifier transition)	2.339	2.040, 2.020, 2.058, 1.994, 2.055	2.033	87	1.3
Low (qualifier transition)	0.0118	0.0088, 0.0085, 0.0083, 0.0083, 0.0086	0.0085	78	2.4
High (qualifier transition)	2.339	2.055, 2.024, 2.076, 2.042, 2.073	2.054	88	1.1

Accuracy and precision / repeatability

The accuracies, reported as mean recovery and precision / repeatability as relative standard deviation are shown in table 1 above. Mean recoveries for each level are in the range 70-110%, the RSD is < 20% per level. The results fulfill the criteria of SANCO/3029/99 rev.4.

Linearity

Linearity was demonstrated for matrix-matched calibrations (8 concentration levels) in the range of 4 to approximately 1200 µg/L a.i. in soil extract for both analytes, with a correlation coefficient (r) greater than 0.99. This is equivalent to 0.005 to 2.8 mg/kg in dry soil (highest validation concentration diluted 2x).

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e., 0.012 mg/kg zoxamide and cymoxanil, per soil dry weight.

Limit of detection

The limit of detection, calculated as three times the noise / blank response, was 0.0009 mg/kg d.w. for zoxamide and 0.0015 mg/kg d.w. for cymoxanil.

Matrix effects

Matrix-matched calibration was used to compensate possible matrix effects.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two mass transitions per analyte for quantification and confirmation. No interfering peaks >30% of LOQ were detected for both mass transitions.

Storage stability of sample extracts and solvent standards

The max. storage period of sample extracts and standard solution was 4 days. Recovery experiments with spiked extract matrix demonstrated storage stability of sample extracts and standard solutions for at least 4 days at 4-8°C.

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 595 days. The freezer storage stability of zoxamide and cymoxanil residues in soil samples was confirmed for 595 days.

Table A 92: Characteristics of the analytical method used for determination of Zoxamide and cymoxanil in artificial soil

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	8-point calibration with external standard. Individual calibration data presented in the report The calibration was linear over the whole calibration range. Calibration curve equation: $y = 93766.3 x + 1178426$, $r^2 = 0.998$	8-point calibration with external standard. Individual calibration data presented in the report The calibration was linear over the whole calibration range. Calibration curve equation: $y = 2427.85 x + 1421.28$, $r^2 = 0.998$
Calibration range	3.98 to 1206 µg/L zoxamide in soil extract (0.005 - 1.43 mg/kg dry soil)	4.00 to 1211 µg/L cymoxanil in soil extract (0.005 – 1.43 mg/kg dry soil)
Assessment of matrix effects	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of quantification (LOQ)	0.012 mg/kg dry soil	0.012 mg/kg dry soil
Limit of detection (LOD)	0.0009 mg/kg dry weight (< 30% of LOQ)	0.0015 mg/kg dry weight (<30% of LOQ)

The following figure shows a representative chromatogram.

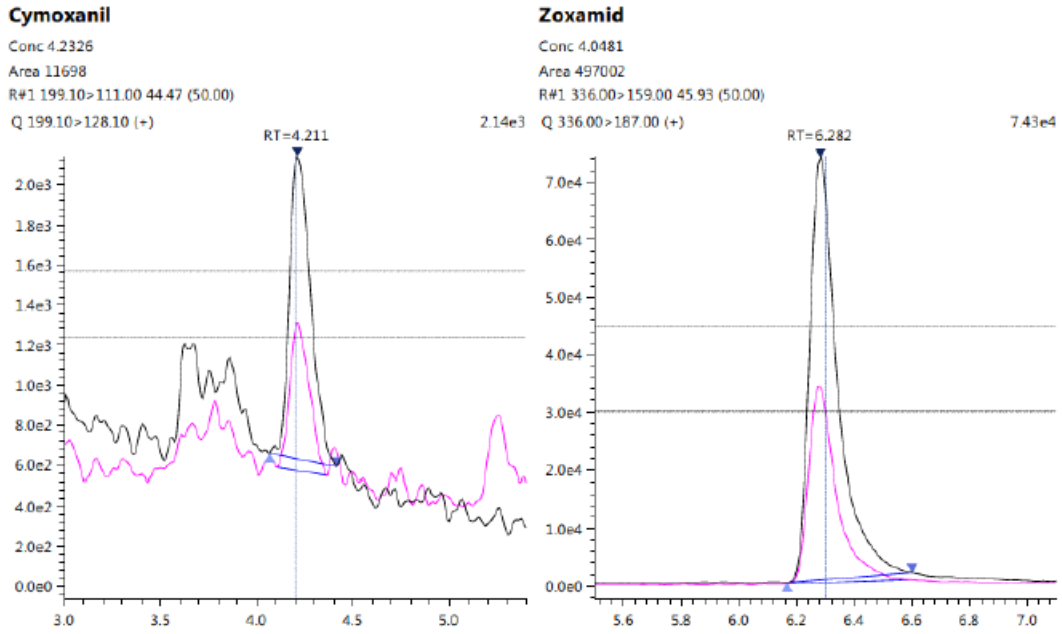


Figure A 44: Chromatogram of the lowest matrix standard

Conclusion

The recoveries in the spray targets (plastic cups filled with 500 g dry soil) were between 74% and 126% after each application. The recoveries in the 0-5 cm soil layer after the first application were 102% and 84% for zoxamide and 101% and 79% for cymoxanil.

The concentrations in the 5 – 20 cm layer were low for zoxamide (maximum mean concentration 0.024 mg/kg in the high treatment after the 5th application) and non-detectable for cymoxanil.

(Schulz L. 2020)

A 2.1.1.7.3.4 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/59**
Report	Parsons, Ch., 2020: Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-3, 18 35 CRX 0026, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of BioChem agrar was used. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.

Acceptability: Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (BioChem project No. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with test item at concentrations of 129 mg/kg a.i. in moist soil (161 mg/kg a.i. or 500 mg/kg test item in dry soil) and at 258 mg/kg a.i. in moist soil (323 mg/kg a.i. or 1000 mg/kg test item in dry soil) with 5 replicates per fortification level. This amounts to a working range of approximately 50 - 100% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) was placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4 : 1 mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed. The analytes were determined after extraction with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 →111), one for quantification and one for qualification, respectively.

Equipment:

HPLC System:	A Shimadzu system with a triple quadrupole mass spectrometric detector was used
Column:	ACE Excel3 C18-AR 100 * 2.1 mm, 3 µm
Mobile phase:	A: water containing 1mL/L formic acid and 5 mmol/l ammonium formate B: methanol containing 1 mL/L formic acid and 5 mmol/l ammonium formate

Time [min]	A [%]	B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate:	0.4 mL/min
Retention time:	Cymoxanil approx. 3.2 min Zoxamide approx. 5.4 min
Run time:	9.0 min
Detection:	ESI positive, MRM
Ion mode	Cymoxanil m/z 199.1 → 128.1, 199.1 → 111.0 Zoxamide m/z 336 → 187, 336 → 159

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 93: Analysis results in moist soil

Treatment group, analyte	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	10000	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	10000	<LOQ		<LOQ		<LOQ	
A, cymoxanil	265.6	1000	270.1	102	222.9	84	193.3	73
A, zoxamide	260.8	1000	268.4	103	262.8	101	250.2	96

n.d.: not detected; <LOQ: concentration below validated limit of quantification (129 mg/kg d.w.), areas of detected peaks were actually below 1.5% of the LOQ (equivalent to approx. 2 mg/kg)

Table A 94: Analysis results in dry soil

Treatment group, analyte	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	<LOQ		<LOQ		<LOQ	
A, cymoxanil	332.0	337.6	102	278.6	84	241.7	73
A, zoxamide	326.0	335.5	103	328.5	101	312.8	96

n.d.: not detected; <LOQ: concentration below validated limit of quantification (161 mg/kg d.w.), areas of detected peaks were actually below 1.5% of the LOQ (equivalent to approx. 2.5 mg/kg)

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the tables below.

Table A 95: Re-validation results for cymoxanil

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.2	120.4	93	4.8
High	5	10000	258.4	238.8	92	9.6
Concentration in dry soil						
Low	5	12500	161.5	150.5	93	4.8
High	5	12500	322.9	298.6	92	9.6

Table A 96: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.1	115.9	90	4.3
High	5	10000	258.3	227.7	88	9.0
Concentration in dry soil						

Low	5	12500	161.4	144.9	90	4.3
High	5	12500	322.8	284.6	88	9.0

Linearity

Linearity was demonstrated for solvent calibration curves (5 concentration levels) of zoxamide in the range of 9.6 to 32 µg/L (corresponding to 96 - 320 mg/kg a.s. in moist soil and 120 - 400 mg/kg a.s. in dry soil) and cymoxanil in the range of 9.7 to 32.5 µg/L (corresponding to 97 - 325 mg/kg a.s. in moist soil and 122 - 406 mg/kg a.s. in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 129 mg/kg cymoxanil and zoxamide, respectively, in moist soil, equivalent to 161 mg/kg in dry soil and 13 µg/L in diluted extracts. However, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 1.5 % of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen soil samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated.

Table A 97: Characteristics for the analytical method

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 28970.8x + 27549.4$, $r^2=0.9997$	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 9690.17x + 4540.47$, $r^2=0.9988$
Calibration range	9.6 to 32.0 µg/L in analytical samples (96 to 320 mg/kg in moist soil)	9.7 to 32.5 µg/L in analytical samples (97 to 325 mg/kg in moist soil)
Assessment of matrix effects is presented	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. Validation blank samples had no peaks with areas >1% of the lowest validation samples).	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. No peaks were found in validation blank samples.
Limit of quantification (LOQ)	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.

The following figures show a representative chromatogram.

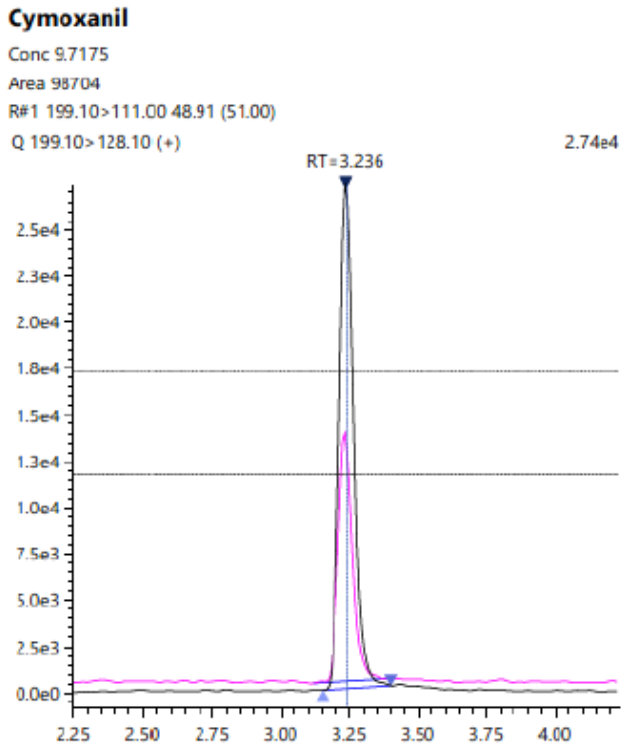


Figure A 45: Chromatogram of cymoxanil

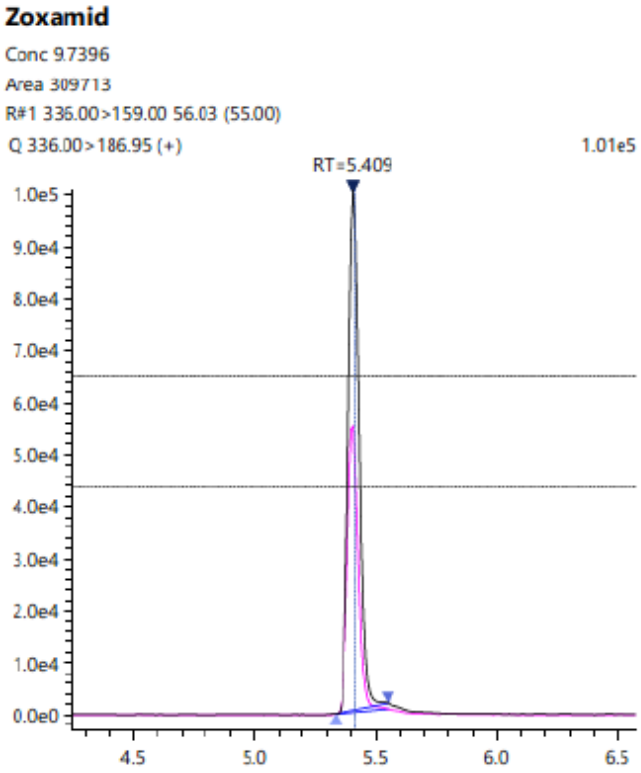


Figure A 46: Chromatogram of zoxamide

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (102% for cymoxanil and 103% for zoxamide). After 28 days, the cymoxanil concentration had declined to 73% of nominal. The zoxamide concentrations stayed within the range of 96 – 103% of nominal throughout the whole study period.

(Parsons Ch. 2020)

A 2.1.1.7.3.5 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/60**
Report	Parson, Ch., 2020: Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-4; 18 35 CRX 0027, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of BioChem agrar was used. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (BioChem project No. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with test item at concentrations of 129 mg/kg a.i.in moist soil (161 mg/kg a.i. or 500 mg/kg test item in dry soil) and at 258 mg/kg a.i. in moist soil (323 mg/kg a.i. or 1000 mg/kg test item in dry soil) with 5 replicates per fortification level. This amounts to a working range of approximately 50 - 100% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugation.

Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed. The analytes were determined after extraction with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 → 111), one for quantification and one for qualification, respectively.

Equipment

HPLC System:	A Shimadzu system with a triple quadrupole mass spectrometric detector was used
Column:	ACE Excel3 C18-AR 100 * 2.1 mm, 3 µm
Mobile phase:	A: water containing 1mL/L formic acid and 5 mmol/l ammonium formate B: methanol containing 1 mL/L formic acid and 5 mmol/l ammonium formate

Time [min]	A [%]	B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate:	0.4 mL/min
Retention time:	Cymoxanil approx. 3.2 min Zoxamide approx. 5.4 min
Run time:	9.0 min
Detection:	ESI positive, MRM
Ion mode	Cymoxanil m/z 199.1 → 128.1, 199.1 → 111.0 Zoxamide m/z 336 → 187, 336 → 159

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 98: Analysis results in moist soil

Treatment group, analyte	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	10000	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	10000	<LOQ		<LOQ		<LOQ	
A, cymoxanil	265.6	1000	230.9	87	216.8	82	202.5	76
A, zoxamide	260.8	1000	233.7	90	246.4	94	244.3	94

n.d.: not detected; <LOQ: concentration below validated limit of quantification (129 mg/kg d.w.), areas of detected peaks were actually below 1% of the LOQ (equivalent to approx. 1.3 mg/kg)

Table A 99: Analysis results in dry soil

Treatment group, analyte	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	<LOQ		<LOQ		<LOQ	

A, cymoxanil	332.0	288.6	87	271.0	82	253.1	76
A, zoxamide	326.0	292.1	90	308.0	94	305.4	94

n.d.: not detected; <LOQ: concentration below validated limit of quantification (161 mg/kg d.w.), areas of detected peaks were actually below 1% of the LOQ (equivalent to approx. 2 mg/kg)

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the tables below.

Table A 100: Re-validation results for cymoxanil

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.2	120.4	93	4.8
High	5	10000	258.4	238.8	92	9.6
Concentration in dry soil						
Low	5	12500	161.5	150.5	93	4.8
High	5	12500	322.9	298.6	92	9.6

Table A 101: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.1	115.9	90	4.3
High	5	10000	258.3	227.7	88	9.0
Concentration in dry soil						
Low	5	12500	161.4	144.9	90	4.3
High	5	12500	322.8	284.6	88	9.0

Linearity

Linearity was demonstrated for solvent calibration curves (5 concentration levels) of zoxamide in the range of 9.6 to 32 µg/L (corresponding to 96 - 320 mg/kg a.s. in moist soil and 120 - 400 mg/kg a.s. in dry soil) and cymoxanil in the range of 9.7 to 32.5 µg/L (corresponding to 97 - 325 mg/kg a.s. in moist soil and 122 - 406 mg/kg a.s. in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 129 mg/kg cymoxanil and zoxamide, respectively, in moist soil, equivalent to 161 mg/kg in dry soil and 13 µg/L in diluted extracts. However, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen soil samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated.

Table A 102: Characteristics for the analytical method

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 28970.8x + 27549.4$, $r^2=0.9997$	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 9690.17x + 4540.47$, $r^2=0.9988$
Calibration range	9.6 to 32.0 µg/L in analytical samples (96 to 320 mg/kg in moist soil)	9.7 to 32.5 µg/L in analytical samples (97 to 325 mg/kg in moist soil)
Assessment of matrix effects is presented	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. Validation blank samples had no peaks with areas >1% of the lowest validation samples).	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. No peaks were found in validation blank samples.
Limit of quantification (LOQ)	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.

The following figures show a representative chromatogram.

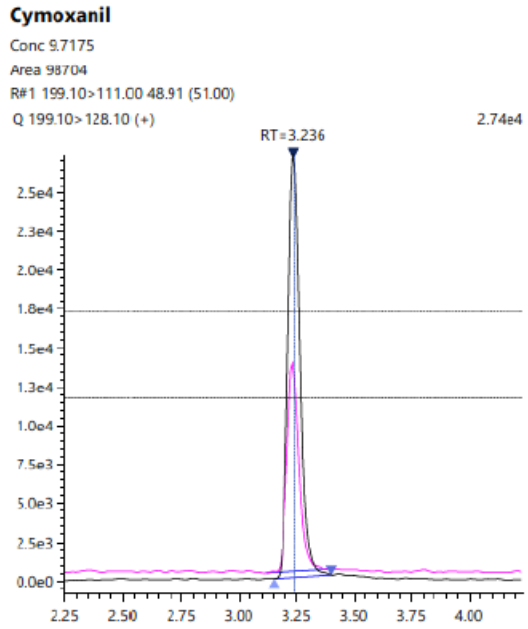


Figure A 47: Chromatogram of cymoxanil

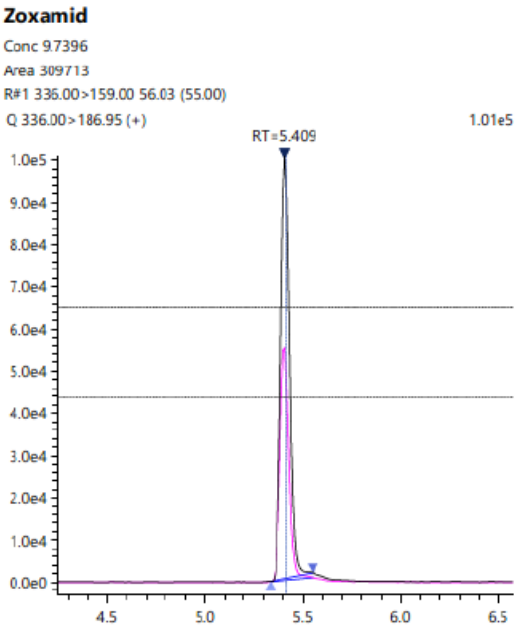


Figure A 48: Chromatogram of zoxamide

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (87% for cymoxanil and 90% for zoxamide). After 14 days, the cymoxanil concentration had declined to 76% of nominal. The zoxamide concentrations stayed within the range of 90 – 94% of nominal throughout the whole study period.

(Parson Ch. 2020)

A 2.1.1.7.4 Analytical method 4 – determination of RH-163353 in soil

The following method has been validated and used for the determination of RH-163353 in artificial soil.

A 2.1.1.7.4.1 Method validation

Comments of zRMS:	Studies 5.1/61, 62, 63 are confirmatory studies for zoxamide currently being evaluated by RMS (Latvia).
Reference:	**KCP 5.1/61**
Report	Gray, J., 2020a: RH-163353: Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202389, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/62**
Report	Gray, J., 2020: RH-163353: Collembolan Reproduction Test in Soil Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202390, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	Each test vessel was weighed and the weight adjusted to the Day 0 level by addition of RO water. In this way individual vessel moisture loss was made up to the correct level whereas the use of a single moisture control vessel as stated in the protocol does not account for the variation in loss between vessels. The process was repeated at Day 21 to ensure optimum environmental conditions were maintained as far as possible throughout the exposure period. The light period was less than 16 hours on one occasion over the 28-day exposure period. These deviations are considered to have no impact on the study integrity.
Acceptability:	Yes

and

Reference:	**KCP 5.1/63**
Report	Gray, J. 2020: RH-163353: Effect on Reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202391, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202389-01V and SMV 3202390-01V and following versions were used to determine the concentration of the analyte RH-163353 in samples of the test media (artificial soil with 5 or 10 % peat) from earthworm and *Folsomia* / *Hypoaspis* studies, respectively. The procedures have

been validated according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-163353 were determined by extracting soil samples with an extraction solvent (1% formic acid in acetonitrile/acetone 3:1 v/v), then diluting further with unfortified control sample extract to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) system:

1. 2 - 5.0 (±0.05) g soil (depending on the residue level) was dispensed into 50 mL Falcon tubes.
2. Samples were fortified as required and shaken well by hand to mix.
3. 20 mL extraction solvent was added.
4. Samples were extracted for a set period of 10 minutes on a rotary shaker set to 200 rpm, sonicated for a set period of five minutes then centrifuged at a set speed of 2500 rpm for 10-15 minutes.
5. If required, samples were filtered through 0.2 µm PTFE syringe filter and/or diluted with unfortified control extract, and a portion of each extract was transferred to a suitable vial for LC/MS analysis.
6. Aliquots of the samples were injected onto the 5600 TOF-MS system.

RH-163353 is a racemate. The analytical method validation for the enantiomeric ratio analysis of RH-163353 was conducted under Smithers ERS Study Number 3202586 (established analytical procedure, SMV 3202586-01V and following) by inclusion of an enantioselective column. A combination of the above-mentioned analytical procedures with SMV 3202586 was used to assess in addition the enantiomeric ratio of the test substance in the test media and calibration standard solution.

Equipment for LC-TOF/MS analysis

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm
Mobile phase:	A: 0.1% Formic acid in water (LC-MS grade) B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	10 µL
Retention time:	2.16 min approx.
Analysis time:	5 min
Ionisation:	Electrospray (ESI), positive
Ion mode:	m/z 332.04 → m/z 186.939-186.989

Equipment for enantiomeric ratio analysis

Instrument:	AB Sciex TripleTOF 5600+ coupled to Shimadzu SIL-30ACMP Quaternary HPLC
Column:	Phenomenex Lux i-Cellulose 5, 150 x 4.6 mm, 5 µm

Mobile phase:	A: 0.1% Formic acid in water (LC-MS grade) B: 0.1% Formic acid in acetonitrile (LC-MS grade) Isocratic elution: %A: 60; %B: 40
Flow rate:	1.0 mL/min
Column temp.:	25°C
Injection volume:	40 µL
Retention time:	Isomer A: 7.42 minutes Isomer B: 8.08 minutes
Analysis time:	15 min
Ionisation:	Electrospray (ESI), positive
Ion mode:	m/z 332.04 → m/z 186.939-186.989

For more detailied information on the MS conditions, please refer to the study report.

Results and discussions

Table A 103: Summary of recovery experiments – earthworm study

10% peat soil	0.016 mg/kg	0.1 g/kg	15 mg/kg	Overall
Mean Recovery (%)	82.5	81.4	83.3	82.4
CV (%)	3.7	2.5	8.0	5.0

Table A 104: Summary of recovery experiments – *Hypoaspis* and *Folsomia* study

10% peat soil	0.16 mg/kg	1.0 g/kg	50 mg/kg	Overall
Mean Recovery (%)	75.8	84.6	82.4	80.9
CV (%)	2.1	2.8	1.0	5.2

Accuracy and precision / repeatability

The accuracy and repeatability / precision of the procedure was determined by fortifying artificial soil medium in quintuplet at three different concentrations. The results for accuracy and precision showed RSD values < 10 % for each spiking level and overall. Recoveries were in the expected range of 70-110%. Untreated samples showed residues < LOD. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by at least 7-points calibration curve (single injections) over the concentration range 0.0005 – 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ was set to 0.016 mg/kg.

Matrix effects

Matrix matched standards were used.

Specificity

The LC-TOF/MS analysis method was considered specific to RH-163353 as the analysis of untreated 10% peat soil samples exhibited no response exceeding 30% of the proposed LOQ of 0.016 mg/kg for RH-163353 from either the test substance or interferences at the retention times of RH-163353.

Storage stability

Freezer storage stability of residues in soil samples has been confirmed over a period of 7 days.

Table A 105: Characteristics of the analytical method validation for the determination of RH-163353 in artificial soil

	RH-163353
Specificity	LC-TOF/MS is regarded specific. Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Matrix matched standard calibration. at least 7-points calibration curve; linear with 1/x weighting; r > 0.99 Calibration data and calibration line equation presented in the study report.
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No. Matrix matched standards used.
Limit of quantification/determination	LOQ: 0.016 mg/kg

The following figure shows a typical chromatogram.

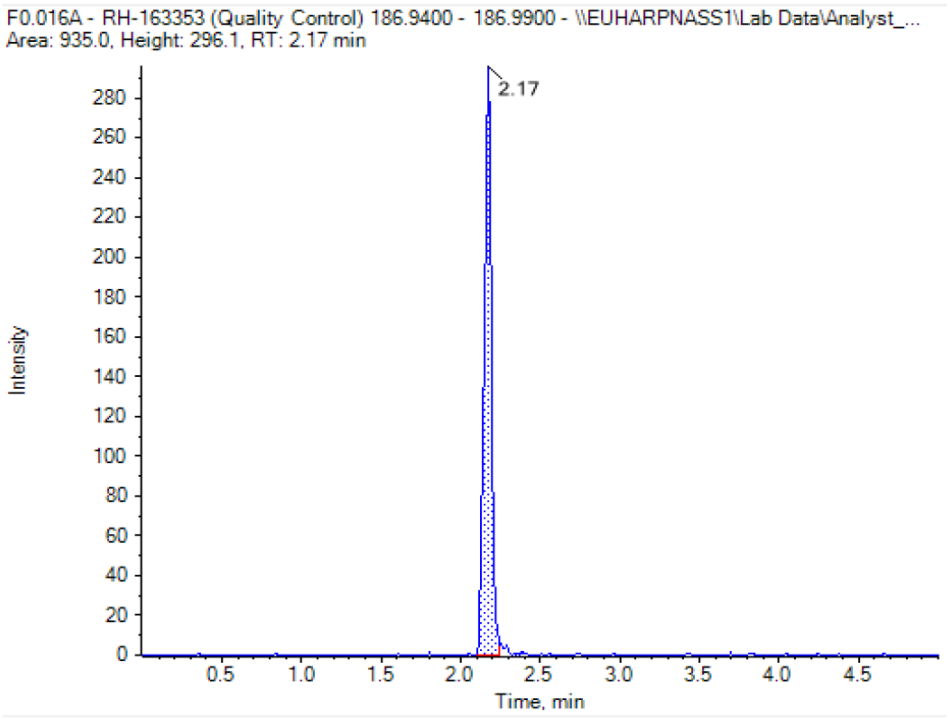


Figure A 49: Representative chromatogram of soil fortified with RH-163353 at 0.016 mg/kg

The mean enantiomeric ratio of RH-163353 on day 14 samples (*Hypoaspis* study) was 47.31% Isomer A and 52.69% of Isomer B. The mean enantiomeric ratio of RH-163353 on day 28 samples (*Folsomia* study) was 47.93% Isomer A and 52.07% Isomer B in comparison to means of 48.91% and 51.09% of Isomers A and B respectively in the calibration samples. The enantiomeric ratio on the Certificate of Analysis is

48.6:51.4. The enantiomeric ratio observed in the samples did not differ from the certificate of analysis value by more than ±1.5%, and was therefore deemed to have not changed during the lifetime of the tests.

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4.

The enantiomeric ratio of RH-163353 was stable in the soils during the lifetime of the tests.

(Gray J. 2020 a, b, c)

A 2.1.1.7.5 Analytical method 5 – determination of RH-127450 in soil

A 2.1.1.7.5.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/64**
Report	Gray, J., 2020: RH-127450: Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Limited, UK, Report No.3202376, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	On one occasion the minimum temperature recorded was 17.8°C, below the guideline minimum of 18°C. The number of juveniles in replicates E-H inclusive of the solvent control was not assessed until day 57 as the assessment took longer than expected due to the high numbers of juveniles recorded. This did not affect the number of juveniles present as the adults had been removed at day 28. However, as all the validity criteria were met, these deviations were not considered to have had any impact on the integrity or outcome of the study.
Acceptability:	Yes

Materials and methods

An analytical method (SMV 3202376-03V) for the determination of RH-127450 in artificial soil (containing 10 % peat) has been validated according to SANCO 3029/99 rev. 4 (2000).

5.0 g soil was dispensed into 50 mL Falcon tubes. Samples were fortified as required and shaken well by hand to mix. 20 mL of MeCN was added and the sample extracted by shaking on a rotary shaker, at a set speed of 200 rpm and for a set period of 10 minutes. This was then sonicated for a set period of five minutes before being centrifuged at a set speed of 2500 rpm for a set period of 15 minutes. A portion of the supernatant was transferred to a suitable vial for LC/MS analysis. If required samples were diluted with unfortified control extract to bring the response within the calibration range. Aliquots of the samples were injected onto a liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) system.

Equipment for LC-TOF/MS Analysis

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software

Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 5 µL
Retention time: 2.28 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 302.07 → m/z 186.9500-186.9900

For further MS conditions, please refer to the study report.

Results and discussions

Table A 106: Summary of recovery experiments

1% peat soil	0.016 µg/mL	0.5 µg/mL	15 µg/mL	Overall
Mean Recovery (%)	99.0	96.9	94.7	96.9
CV (%)	3.72	1.22	0.785	2.87

Accuracy and precision / repeatability

The accuracy and repeatability / precision of the procedure was determined by fortifying 5 g aliquots of 10% peat soil medium (in quintuplet) at three different concentrations. The results showed RSD values of < 10 % for each spiking level and the soil. Recovery values were within the required range of 70-110 %. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by 7-points calibration curve (single injections) using matrix matched standards at a range of 0.0005 – 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.016 mg/kg.

Matrix effects

Matrix matched standards were used for calibration.

Specificity

The LC-TOF/MS analysis method was considered specific to RH-127450 as the analysis of untreated 10% peat soil samples exhibited no interferences exceeding 30% of the proposed LOQ of 0.016 mg/kg at the retention time of RH-127450.

Storage stability

Freezer storage stability of RH-127450 residues in 10% peat soil was confirmed for at minimum 7 days.

Table A 107: Characteristics of the analytical method for the determination of RH-127450 in artificial soil

	RH-127450
Specificity	The LC-TOF/MS method is considered specific. Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Matrix matched calibration. 7 points calibration; linear with 1/x weighting. Correlation coefficient r >0.99. Calibration data and calibration line equation presented in the study report.
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No. Matrix matched standard calibration.
Limit of quantification (LOQ)	LOQ: 0.016 mg/kg

The following figure shows a typical chromatogram.

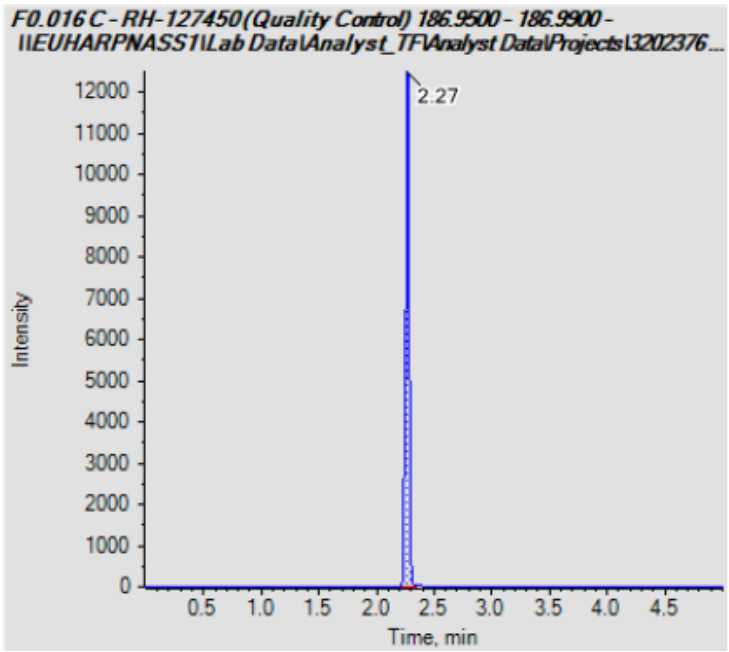


Figure A 50: **Chromatogram - 10% peat soil fortified with RH-127450 at 0.016 mg/kg**

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4 (2000).

(Gray J. 2020)

A 2.1.1.7.6 Analytical method 6 – determination of RH-24549 in soil

A 2.1.1.7.6.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/65**
Report	Gray, J., 2019: RH-24549 - Effect on reproduction in the earthworm *Eisenia fetida* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202395, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	The light intensity deviated from the guideline times of 16:8 hours light:dark on one occasion during the reproduction phase of the test, when the timer was still recording a dark period after 15.05 hours. The soil used in the study had a peat content of 8.1% and not 10% as stated in the protocol. The pipette used to adjust the soil moisture content on day 35 (06 August), day 42 (13 August 2019) and day 49 (20 August 2019) was found to have failed to meet the required criteria when calibrated on 27 August 2019. These deviations were not considered to have had an adverse impact on the study as all the validity criteria were met.
Acceptability:	Yes

Materials and methods

An analytical method (SMV 3202395-01V, and later revisions) was used to confirm the concentrations of RH-24549 in artificial soil samples (10 % peat). The method was validated according to SANCO 3029/99 rev. 4.

5.0 g soil was dispensed into 50 mL Falcon tubes. Samples were fortified as required and shaken well by hand to mix. 20 mL of extraction solvent (1% formic acid in acetonitrile) was added and the sample extracted by shaking on a rotary shaker, at a set speed of 200 rpm for a set period of 10 minutes. This was then sonicated for a set period of five minutes and centrifuged at a set speed of 2500 rpm for a set period of 15 minutes. A portion of the supernatant was transferred to a suitable vial for LC/MS analysis. Where required, the sample was diluted with unfortified control extract to bring the response within the calibration range. Samples were analysed by injection via liquid chromatography with time-of-flight mass spectrometry (LC-TOF/MS) or liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system.

Equipment for LC-TOF/MS analysis

Instrument:	AB Sciex TripleTOF5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm

Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 10 µL
Retention time: 2.16 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), negative
Ion mode: m/z 202.97 → m/z 158.953-159.003

Equipment for LC-TQMS analysis

Instrument: API 5000 TQMS coupled to Shimadzu SIL-30ACMP Quaternary HPLC
Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
0.50	95	5
2.50	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 3 µL
Retention time: 2.28 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 202.9 → m/z 158.9

For further mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 108: Summary of recovery experiments

10% peat soil	0.016 mg/kg	0.5 g/kg	10 mg/kg	Overall
	LC-TOF/MS			
Mean Recovery (%)	87.9	86.5	89.2	87.9
CV (%)	1.99	3.02	1.63	2.46
	LC-TQMS			
Mean Recovery (%)	98.8	99.8	96.6	98.4

CV (%)	2.5	3.7	4.4	3.6

Accuracy and precision / repeatability

The accuracy and precision of the procedure were determined by fortifying 5 g aliquots of 10% peat soil medium in quintuplet at three different concentrations. The results showed RSD values of < 10 % for each spiking level and the soil. Recovery values were within the required range of 70-110 %. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by an 8-points calibration curve (single injections) over a concentration range of 0.0005 – 0.1 µg/mL using matrix matched standard solutions. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.016 mg/kg.

Matrix effects

Matrix matched standards were used for calibration.

Specificity

Both methods were considered specific to RH-24549 as the analysis of untreated 10% peat soil samples exhibited no interferences exceeding 30% of the proposed LOQ of 0.016 mg/kg for RH-24549 at the retention time of RH-24549.

Storage stability

Freezer storage stability of samples was assessed and confirmed for at minimum 6 days.

Table A 109: Characteristics of the analytical method validation for the determination of RH-24549 in artificial soil

	RH-24549
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. 8-points calibration; linear with 1/x weighting; r >0.99. Calibration data and calibration line equation presented in the study report.
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of determination/quantification	LOQ: 0.016 mg/kg

The following figur shows a typical chromatogram.

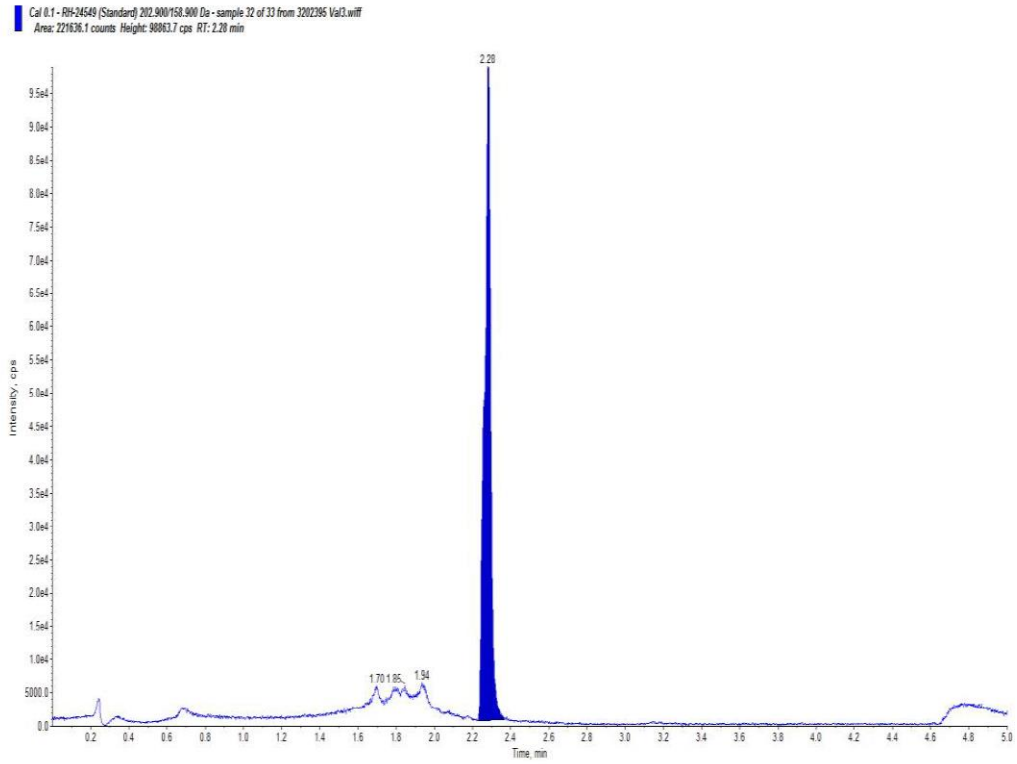


Figure A 51: Representative chromatogram of a calibration standard containing 0.1 µg/mL RH-24549 in unfortified control extract using LC-TQMS

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4 (2000).

(Gray J. 2019)

A 2.1.1.7.7 Analytical method 7 – determination of RH-141455 in soil

The following method has been validated and used for the determination of RH-141455 in artificial soil.

A 2.1.1.7.7.1 Method validation

Comments of zRMS:	Studies 5.1/66 and 67 are confirmatory studies for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/66**
Report	Gray, J., 2020: RH-141455: Collembola reproduction test in soil Gowan Crop Protection Ltd., UK Smithers ERS Limited, UK., Report No. 3202382, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/67**
Report	Gray, J., 2020: RH-141455: Effect on reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* Gowan Crop Protection Ltd., UK Smithers ERS Limited, UK, Report No.3202383, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	The light period was less than the 16 hours specified. The analytical data confirmed that the applied dose was in excess of the required 50 mg a.i./kg but showed it was also outside the accepted range of 80-100% of nominal (+29.28% at Day 0 and + 50.01% at Day 14). However, as the dose was in excess of that required it is considered that this confirms no adverse effect on survival or reproduction at the intended dose. As all the validity criteria were met. Therefore, these deviations were not considered to have had any impact on the integrity or outcome of the study.
Acceptability:	Yes

Materials and methods

The same analytical procedure (SMV 3202383-01V and following versions) was used to determine the concentration of the analyte RH-141455 in samples of the test medium (artificial soil) from *Folsomia* and *Hypoaspis* studies. The procedures have been validated according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-141455 were determined by extracting soil samples with an extraction solvent, then diluting further with unfortified control sample extract to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography LC-FT/MS or LC-TOF/MS system :

1. 2.0 (± 0.05) g soil was dispensed into 50 mL Falcon tubes.
2. Samples were fortified as required and shaken well by hand to mix.
3. 40 mL extraction solvent (acetonitrile/acetone 2:1, v/v containing 1% formic acid) was added.
4. Samples were extracted on a rotary shaker set to 200 rpm for a set period of 10 minutes, sonicated for a set period of five minutes then centrifuged at a set speed of 2500 rpm for 5 minutes.
5. An aliquot of the final extract was filtered through 0.2 µm PTFE syringe filter and diluted if required with filtered unfortified control extract. A portion of the extract was transferred to a suitable vial for LC/MS analysis.
6. Aliquots of the samples were injected onto the ThermoScientific Q-Exactive or the 5600 TOF-MS system.

Equipment for LC-FT/MS analysis

Instrument:	Thermo Scientific Q-Exactive Coupled to Shimadzu SIL-30AC Quaternary HPLC system. Xcalibur 4.1SP2 data collection software
Column:	Scherzo SM-C18, 3µm, 50 x 2.0mm

Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
3.00	0	90
3.90	0	90
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 50 µL
Retention time: 4.5 min approx.
Analysis time: 5 min
Ionisation: Heated Electrospray (HESI), negative
Ion mode: m/z 232.94 → m/z 188.953-189.003

Equipment for LC-TOF/MS analysis

Instrument: AB Sciex Triple TOF 5600+ Coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column: Scherzo SM-C18, 3.0 µm, 50x 2.0 mm
Mobile phase: A: 0.1% Formic acid in water (LC-MS grade)
B: 0.1% Formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
3.00	0	100
3.90	0	100
4.00	95	5
5.00	95	5

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 25 µL
Retention time: 3.8 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), negative
Ion mode: m/z 232.95 → m/z 188.953-189.003

For further mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 110: Summary of recovery experiments

Artificial soil	LC-FT/MS			
	0.2 mg/kg	1.0 g/kg	75 mg/kg	Overall
Mean Recovery (%)	106	102	94.6	101
CV (%)	3.80	3.58	2.33	5.64
	LC-TOF/MS			

Mean Recovery (%)	112	116	104	111
CV (%)	3.01	2.64	2.19	5.22

Accuracyand precision / repeatability

The accuracy and precision of the procedure were determined by fortifying aliquots of artificial soil medium in quintuplet at three different concentrations. The results showed RSD values of < 10 % for each spiking level and the soil. Recovery values were within the required range of 70-110 %. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by 8-points calibration curve (single injections) using matrix matched standards at a range of 0.001 to 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.2 mg/kg.

Matrix effects

Matrix matched standards were used.

Specificity

Both methods were considered specific to RH-141455 as the analysis of untreated artificial soil samples exhibited no interferences > 30% of the proposed LOQ from the matrix at the retention time of the analyte.

Storage stability

Freezer storage stability of RH-141455 residues in soil samples has been assessed to be at minimum 29 days.

Table A 111: Characteristics of the analytical method for the determination of RH-141455 residues in artificial soil

	RH-141455
Specificity	Both methods (LC-FT/MS and LC-TOF/MS) are considered specific. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. 8-points calibration curve (single injections); linear with 1/x weighting; r > 0.99. Calibration data and calibration line equation presented in the study report.
Calibration range	0.001 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix matched stanstards were used).
Limit of quantification/determination	LOQ: 0.2 mg/kg

The following figure shows a typical chromatogram.

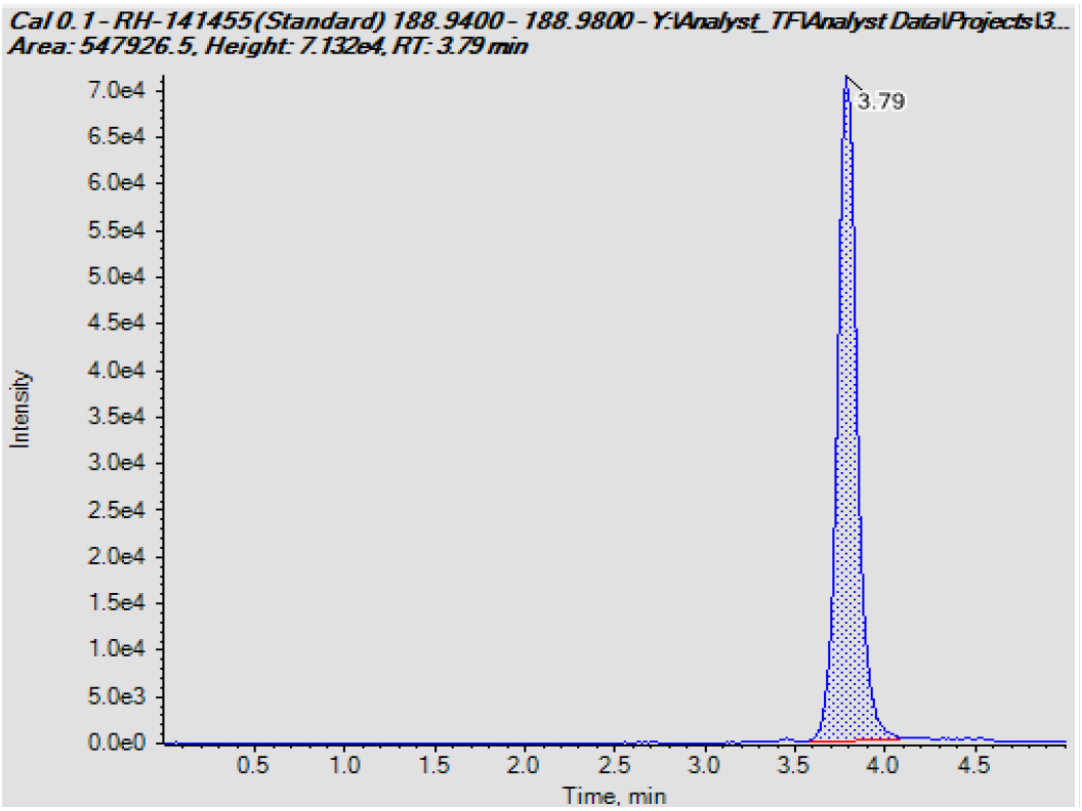


Figure A 52: Chromatogram of a of calibration standard containing 0.1 µg/mL RH-141455 in unfortified control extract using LC-TOF/MS

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev.4.

(Gray J. 2020)

A 2.1.1.8 Description of analytical method for the determination of residues in body fluids and tissues

A 2.1.1.8.1 Analytical method 1

The following method U-19104 to estimate residues of RH-141455 in blood plasma of rats has been validated under GLP conditions in study report no. U-19102 (90 days dietary toxicological study with RH-141455 in rats). The method was applied in study report no. U-19044 (non GLP 2-day oral dietary pharmacokinetic study with RH-141455 in rats) and U-19071 (the 14 days dietary toxicological study with RH-141455 in rats).

A 2.1.1.8.1.1 Method validation

Comments of zRMS:	Studies 5.1/68, 69, 70 are confirmatory studies for zoxamide currently being evaluated by RMS (Latvia).

Reference: **KCP 5.1/68**

Report	xxx, 2020: RH-141455: 90-day oral dietary toxicity study with toxicokinetics and 28-day recovery period in Sprague Dawley rats Gowan Crop Protection Ltd., UK xxx, Report No. U-19102, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/69**
Report	xxx, 2019: RH-141455: 2 days oral dietary pharmacokinetic study in Sprague Dawley Rats Gowan Crop Protection Ltd., UK xxx, Report No. U-19044, No GLP, Not published
Guideline(s):	None (investigative study)
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/70**
Report	xxx, 2020: RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats xxx., UK xxx, Report No. U-19071, No GLP, Not published
Guideline(s):	None (investigative study)
Deviations:	The blood collection for toxicokinetics on day 2 (6 P.M) & day 3 (6 A.M) and on day 14 (6 A.M) was delayed between 1- 18 minutes from the scheduled time (± 10 minutes) across the groups. However, this deviation is regarded to have no impact on the integrity of the study.
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-141455 in rat blood plasma.

RH-141455 and an internal standard (tolbutamide) were extracted from Sprague Dawley rat blood plasma using protein precipitation as extraction technique. The extracted plasma samples were analysed by LC-MS/MS. The concentration of samples was calculated by constructing a calibration curve plotting peak area ratio of RH-141455 and internal standard versus concentration by applying best fit linear regression equation with $1/X^2$ weighting factor.

Deep frozen plasma samples were allowed to thaw. A completely thawed sample was vortexed at 2000 rpm for 5 minutes. Aliquots of 25µL were pipetted in pre-labelled polypropylene tubes. 25 µL internal standard

working solution (tolbutamide diluted in acetonitrile) was added to all tubes except the standard blank sample (only 25 µL acetonitrile). In each tube 500 µL of precipitation solvent was added (mobile phase B, 0.1% formic acid in acetonitrile) and vortexed at 2000 rpm for 5 minutes. Centrifugation was carried out at 4000 rpm for 5 minutes. Samples were transferred into pre-labelled auto-sampler vials and loaded onto LC-MS/MS for analysis.

Equipment (HPLC system) for RH-141455

Instrument:	Shimadzu Nexera
Column:	X-Bridge C18 4.6*100, 5 µm
Column temp.:	45 °C
Mobile phase:	A: Milli Q water B: 0.1% (v/v) formic acid in acetonitrile

Time [min]	% A	% B
0.01	30	70
3.0	System controller stop	

Flow rate:	0.900 mL/min
Injection volume:	10 µL
Run time:	3 minutes
Retention time:	approx. 1.4 minutes (RH-141455 and tolbutamide)

LC-MS/MS Parameters

Instrument	API 4000 Triple Quadrupole
Source	Turbo Ion Spray
CAD Gas	7 L / Minute
Curtain Gas	20 L / Minute
Ion Source Gas (GS1)	45 L / Minute
Heater Gas (GS2)	50 L / Minute
Ion Spray Voltage	-4500 Volt
Temperature	450°C
Interface Heater	On

Compound Dependent Parameters	**RH-141455**	**Tolbutamide**
Ionization Mode	Negative	Negative
Q1 Mass (amu)	233.0	269.3
Q3 Mass (amu)	188.8	170.2
De-clustering Potential (Volts)	-55	-80
Entry Potential (Volts)	-10	-10
Collision Energy (Volts)	-13	-30
Collision Cell Exit Potential (CXP)	-11	-10
Dwell Time (milli seconds)	200	200

Results and discussions

Table A 112: Summary of recovery experiments (and extraction efficiency)

Level	Recovery Samples (Extracted)					Post extracted (comparison samples)			
	Peak Area	Mean peak area	SD	% RSD	Mean % recovery	Peak area	Mean peak area	SD	% RSD
LQC	8546	8415	438	5.20	59.23	11190	14207	1631	11.48
	7815					15365			
	8775					13643			
	7952					14530			
	8509					15597			
	8894					14915			
MQC	539985	543887	31618	5.81	63.36	780514	858342	51061	5.95
	491561					834662			
	530829					906313			
	557359					846308			
	558842					860943			
	584748					921313			
HQC	1059289	1088584	31490	2.89	62.89	1609861	1730871	102287	5.91
	1085206					1647445			
	1130324					1695589			
	1121303					1727869			
	1081878					1851625			
	1053502					1852834			
ISTD	845670	843457	13389	1.59	85.13	957231	9904798	24330	2.46
	867571					984090			
	843944					1011248			
	838727					969401			
	827750					1016993			
	837080					1005825			
Mean Global % Recovery (RH-141455)									61.83
% RSD									3.66

Table A 113: Summary of intra run precision and accuracy for RH-141455

Parameter	Results			
Intra Run Precision & Accuracy	**Analyte**	**QC Lever**	**%RSD**	**%RE**
(%Bias, ±20 %, % RSD ≤20% at LOQQC level) (Bias, ±15%, % RSD ≤15% at remaining QC)	RH-141455	LOQQC	6.03 to 8.04	-1.92 to 11.54
		LQC	2.95 to 6.87	-1.69 to 3.04
		MQC	5.84 to 7.14	-0.26 to 1.84
		HQC	2.57 to 3.69	0.93 to 6.23
(%Bias, ±20 %, % RSD ≤20% at LOQQC level) (Bias, ±15%, % RSD ≤15% at remaining QC)	RH-141455	LOQQC	9.09	5.77
		LQC	5.39	-0.34
		MQC	6.04	0.79
		HQC	3.74	3.86

Accuracy and precision / repeatability

Recovery of RH-141455 in rat blood plasma was evaluated at LQC (low quality control), MQC (mid quality control) and HQC (high quality control) levels. The % recovery of RH-141455 was consistent at all three

levels. The overall recovery of RH-141455 was 61.83% with a RSD of 3.66%. The internal standard recovery was calculated at MQC level and was found 85.13 % with a RSD of 1.59%. The recovery of the internal standard was therefore within the required range of 70-110%.

The results for accuracy and precision showed RSD values < 10%. The accuracy and precision were measured by % bias, which was determined by comparing the mean values of the measured concentrations with the nominal concentrations. The accuracy of RH-141455 was determined at LOQQC (lower limit of quantification), LQC (low quality control), MQC (mid quality control) and HQC (high quality control) level in six replicates during the validation on two different days.

The intra run/ intra-day accuracy as %bias (RE %) at QC levels varied from -1.92% to 11.54% at LOQQC, -1.69% to 3.04% at LQC, -0.26% to 1.84% at MQC and 0.93% to 6.23% at HQC level, respectively.

The inter-run/ inter-day accuracy for LOQQC, LQC, MQC and HQC were 5.77%, - 0.34%, 0.79%, and 3.86% respectively for RH-141455.

Linearity

The weighting factor was calculated for three different precision and accuracy batches. The best fit was weighted as linear regression with $1/x^2$ weighting factor. The linearity of the method was determined by a weighted ($1/x^2$) least square regression analysis of standard plots associated with a 10-point calibration curve for RH-141455. The calibration line was linear for the standards ranging from 0.100 µg/mL to 49.949 µg/mL for RH-141455, which were injected in the beginning analytical run (r^2 >0.98). These calibration curves were used to determine the concentration of validation samples.

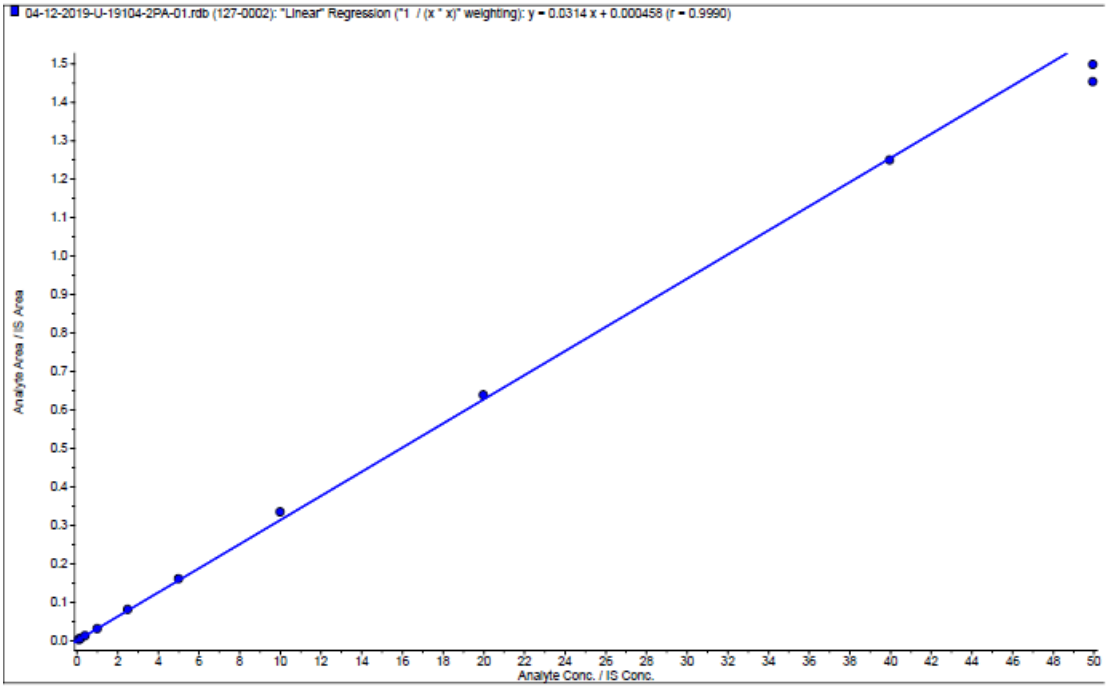


Figure A 53: Typical calibration curve for RH-141455

Limit of quantification

The LOQ of the method was defined at 0.104 µg/mL (= lower limit of quantification (LOQQC)).

Matrix effects

Matrix effects for RH-141455 and tolbutamide were calculated, both for the internal standard and RH-141455.

Specificity

The LC-MS/MS method was regarded specific for the analytes. No significant interference was observed at the retention time of RH-141455 and tolbutamide in blank and blank with internal standard samples (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

Storage stabilities were tested.

RH-141455 was found stable up to 99 days at -70°C ± 10°C and 94 days at -20°C ± 5°C in plasma.

RH-141455 was found to be stable in blood plasma at room temperature for at minimum 6.65 hours.

The stability of RH-141455 in plasma samples during freeze-thaw cycles (3 cycles) was confirmed.

Auto-sampler stability (10°C) of RH-141455 in plasma samples was found to be at least for 51.08 hours.

Stock solutions and working solutions of RH-141455 and tolbutamide were stable for at least 6.38 hours at room temperature.

Table A 114: Characteristics for the analytical method validation for the determination of RH-141455 in rat blood plasma

	RH-141455
Specificity	Typical chromatograms provided. LC-MS/MS method is regarded specific. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 10-point calibration, linear with 1/x weighting. Correlation coefficient r^2 was > 0.98 Individual calibration data presented in the study report.
Calibration range	0.100 µg/mL to 49.949 µg/mL
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ = 0.104 µg/mL

Typical chromatograms are presented in the report.

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-141455 in rat blood plasma.

RH-141455 was found stable up to 99 days at -70°C ± 10°C and 94 days at -20°C ± 5°C in plasma.

(xxx 2019, 2020 a,b)

A 2.1.1.8.2 Analytical method 2

A 2.1.1.8.2.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference: **KCP 5.1/71**

Report	xxx, 2020: RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats Gowan Crop Protection Ltd., UK xxx, Report No. U-19134, No GLP, Not published
Guideline(s):	No. (Laboratory internal SOP)
Deviations:	The draft report was provided later to the sponsor than intended by the study plan. However, this deviation does not have an impact on the integrity of the study.
Acceptability:	Yes

Materials and methods

The objective of this part of the study was to determine the concentrations of RH-150721 and metabolite (RH-141455) in K_2EDTA Sprague Dawley rat plasma samples. They were analysed as per study plan using a fit for purpose LC-MS/MS method as a non GLP activity. The method for the determination of RH-150721 and RH-141455 via LC-MS/MS has been validated according to laboratory SOP using an internal standard (tolbutamide). As a result, neither RH-150721 nor RH-141455 (as one possible transformation product of RH-150721) could be observed (< LOD) in the blood plasma samples of the pharmacokinetic rat study.

K_2EDTA rat blood plasma was separated by centrifuging the whole blood sample. After extraction and centrifugation, the supernatant was transferred into auto sampler vials and volumes of 10 µL were injected on a HPLC column with LC-MS/MS detection. The samples were analysed using a calibration curve range from 0.064 µg/mL to 50.370 µg/mL (matrixed matched standards).

Equipment (HPLC system) for RH-150721

Instrument:	Shimadzu Nexera
Column:	X-Bridge 4.6*100, 5 µm
Column temp.:	45 °C
Mobile phase:	A: Type-I water B: 0.1% Formic acid in Acetonitrile, v/v

Time [min]	% A	% B
0.01	25	75
3.0	25	75

Flow rate:	0.800 mL/min
Injection volume:	5 µL
Run time:	3 minutes
Retention time:	approx. 1.0 min

For API 4000 Triple Quadrupole conditions, please refer to the study report.

Compound Dependent Parameters	RH-150721	Tolbutamide
Ionization Mode	Positive	Positive
Q1 Mass (amu)	381.1	271.3
Q3 Mass (amu)	187.0	91.1
De-clustering Potential (Volts)	80	50
Entry Potential	10	10
Collision Energy	30	42

Collision Cell Exit Potential (CXP)	20	10
Dwell Time	200	200

Equipment (HPLC system) for RH-141455

Instrument:	Shimadzu Nexera
Column:	X-Bridge C18 4.6*100, 5 µm
Column temp.:	45 °C
Mobile phase:	A: 0.1% ammonium acetate in Milli Q water B: Acetonitrile

Time [min]	% A	% B
0.01	30	70
3.0	30	70

Flow rate:	0.900 mL/min
Injection volume:	10 µL
Run time:	4 minutes
Retention time:	approx. 1.4 minutes

For API 4000 Triple Quadrupole conditions, please refer to the study report.

Compound Dependent Parameters	RH-141455	Tolbutamide
Ionization Mode	Negative	Negative
Q1 Mass (amu)	233.0	269.3
Q3 Mass (amu)	188.8	170.2
De-clustering Potential (Volts)	-55	-80
Entry Potential	-10	-10
Collision Energy	-13	-30
Collision Cell Exit Potential (CXP)	-11	-10
Dwell Time	200	200

The following acceptance criteria of the SOP were followed:

- System suitability: Area ratio of analyte and internal standard ≤ 5 %, Analyte and ISTD RT variation ≤ 5 %.
- In Standard Blank/Standard Zero, response of the interfering peaks, if any, at the retention time of analyte or internal standard should not be more than 20 % of the extracted LLOQ response (CC1) for analyte and 5 % of the mean peak response of the ISTD observed for the passing nonzero CC standards and QC samples.
- r^2 should be > 0.98.
- 20 % deviation of the LLOQ (STD 1) from nominal concentration.
- 15% deviation of the other calibration standards other than LLOQ from nominal concentration.
- Mean % nominal concentration at each quality control sample level must be between 85% and 115% and the precision should be +15% of the %CV.
- Accuracy of at least 67% of total quality control samples and at least 50% quality control samples at each level should meet the above acceptance criteria.

Conclusion

The method calibration data met the SOP acceptance criteria.

(xxx 2020)

A 2.1.1.9 Description of analytical method for the determination of residues in animal feed

A 2.1.1.9.1 Analytical method 1

The following method U-19162 to estimate residues of RH-150721 in rat feed is used in the 14 days and 90 days oral dietary toxicological studies U-19189 and U-19235.

A 2.1.1.9.1.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/72**
Report	xxx 2020: Analytical method validation for the estimation of RH-150721 in rat feed by reverse phase high performance liquid chromatography Gowan Crop Protection Ltd., UK Syngene International Limited, India, Report No. U-19162, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-150721 in rat feed by reverse phase high performance liquid chromatography (HPLC) with UV detection (200 nm) in order to check the homogeneity and stability of the test item in a rat feed preparation. As a result, the analyte was shown to be homogenic and stable in the rat feed for at minimum 2 days for 670 ppm, 4 days for 5000 ppm and 8 days for 16000 ppm RH-150721 in the rat feed (recoveries > 80%).

The required amount of the formulated feed was weighed in a 50 mL centrifuge tube. Methanol was added and the sample was vortexed for 5 minutes. The vortexed content was centrifuged for 5 minutes at 5000 rpm. A volume of 2.0 mL supernatant from the centrifuged samples was transferred into a 10 mL volumetric flask and the volume made up to the mark with diluent (for mid dose). A volume of 1.0 mL supernatant from the centrifuged samples was transferred into 10 mL volumetric flask and the volume made up to the mark with diluent (for high dose).

Equipment (HPLC System)

Instrument:	Agilent/ 1200 Series
Column:	Agilent, Zorbax SB-C18 5µm, 4.6 x 150 mm

Mobile phase: A: 0.1% ammonium acetate in Milli Q water
B: acetonitrile

Time [min]	% A	% B
0	85	15
5	55	45
15	5	95
25	85	15

Flow rate: 1.0 mL/min
Column temp.: 30 °C
Injection volume: 25 µL
Run time: 25 minutes
Retention time: 13.02 Min.

Results and discussions

Table A 115: Summary of recovery experiments

Dose level	Sampling Location	Final Concentration (mg/mL)	% Recovery	Mean % Recovery	SD	% RSD
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose	1	0.100	99.01	98.42	0.54	0.55
	2	0.100	99.01			
	3	0.099	98.02			
	4	0.099	98.02			
	5	0.099	98.02			
High dose	1	0.985	97.91	97.63	0.49	0.50
	2	0.975	97.01			
	3	0.983	97.91			
	4	0.977	97.21			
	5	0.988	98.11			
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose (extended for stability)	1	0.104	102.97	101.39	1.13	1.11
	2	0.101	100.00			
	3	0.103	101.98			
	4	0.102	100.99			
	5	0.102	100.99			
Mid dose	1	0.517	102.58	102.94	0.58	0.56
	2	0.522	103.57			
	3	0.516	102.38			
	4	0.522	103.57			
	5	0.517	102.58			
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose	1	0.124	109.73	108.49	1.01	0.93

Extended	2	0.123	108.85			
	3	0.122	107.96			
	4	0.123	108.85			
	5	0.121	107.08			

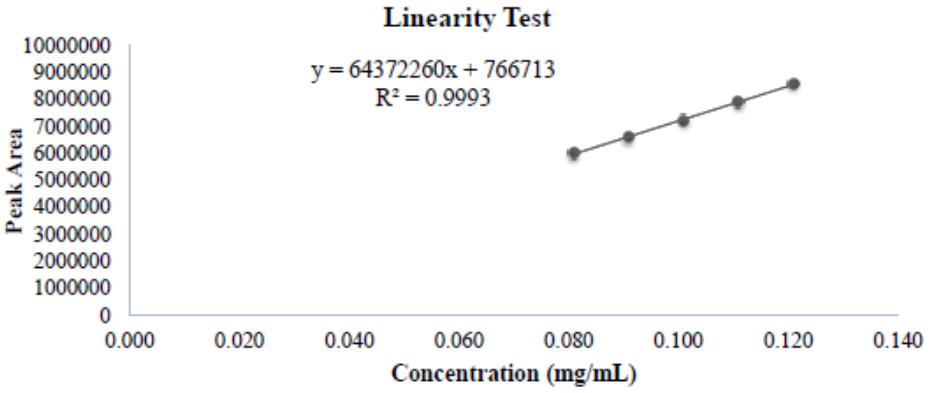
Accuracy and precision / repeatability

The results for accuracy and precision showed RSD values of < 10% for each spiking level and overall and recovery values within the required range of 70—110%. Three untreated samples were analysed with recovery values < LOD.

Linearity

Linearity was determined by 5-points calibration curve (single injections) using matrix matched standards at a range of 0.081 – 0.121 mg/mL ($R^2 > 0.99$). The standard solutions were injected in single replications for each level.

ID	Concentration (mg/mL)	Peak Area
Linearity level-1	0.081	6009371
Linearity level-2	0.091	6612086
Linearity level-3	0.101	7235737
Linearity level-4	0.111	7900666
Linearity level-5	0.121	8583694



Limit of quantification

Based on the available data, the LOQ was estimated to be 0.091 mg/mL.

Limit of detection

Based on the available data, the LOD was estimated to be 0.081 mg/mL.

Matrix effects

Matrix-matched standard solutions were used.

Specificity

The method was regarded specific with a stable retention time of the analyte of 13.02 min. No interference of the matrix was observed at the retention time of analyte (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

No information given. However, high recoveries demonstrated the storage stability of the samples and sample extracts.

Table A 116: Characteristics for the analytical method validation for the determination of RH-150721 in rat feed

	RH-150721
Specificity	Typical chromatograms provided. Stable retention time of the analyte of 13.02 min. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 5-point calibration, linear. y = 64372260x + 766713 Correlation coefficient r^2 was > 0.99 Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.081 mg/mL – 0.121 mg/mL
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ was estimated to be 0.091 mg/mL LOD was estimated to be 0.081 mg/mL

The following figure shows a typical chromatogram.

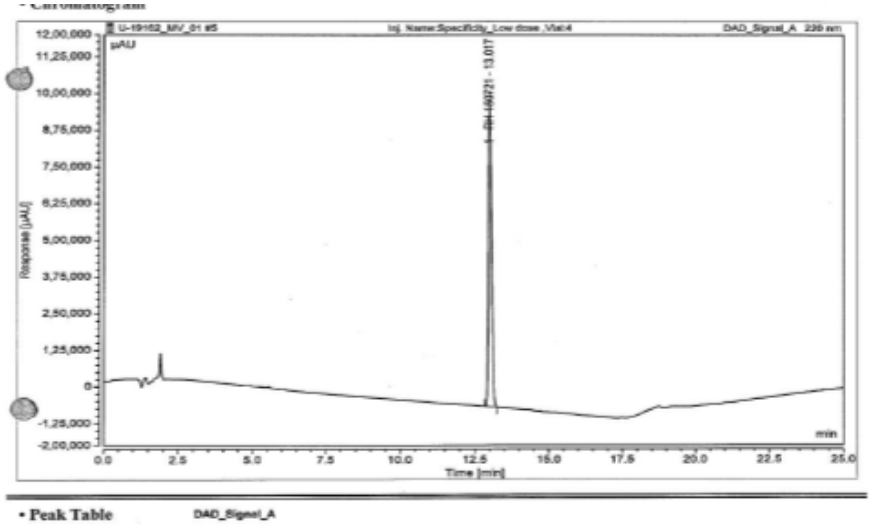


Figure A 54: **Typical Chromatogram of RH-150721**

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-150721 in rat feed.

(xxx 2020)

A 2.1.1.9.2 Analytical method 2

The following method U-19069 to estimate residues of RH-141455 in rat feed is used in the 14 days and 90 days oral dietary toxicological studies U-19071 and U-19102.

A 2.1.1.9.2.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/73**
Report	xxx, 2019: RH-141455: Analytical method validation for the estimation of RH-141455 in rat feed by reverse phase high performance liquid chromatography was used for the dose formulation analysis Gowan Crop Protection Ltd., UK xxx, Report No. U-19069, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-141455 in rat feed by reverse phase high performance liquid chromatography (HPLC) with UV detection (200 nm) in order to check the homogeneity and stability of the test item in a rat feed preparation. As a result, the analyte was shown to be homogenic and stable in the rat feed for at minimum 8 days with recoveries of 92.22-115.35% of nominal.

The required amount of the formulated feed was weighed in a 50 mL centrifuge tube, extraction solution was added and the sample was vortexed for 5 minutes. The vortexed content was centrifuged for 10000 rpm at 5 minutes. A volume of 1.0 mL supernatant from the centrifuged samples was transferred into a 10 mL volumetric flask and the volume made up to the mark with diluent (for low dose). A volume of 0.1 mL supernatant from the centrifuged samples was transferred into 10 mL volumetric flask and the volume made up to the mark with diluent (for high dose). Control feed was diluted as per low dose preparation. The dissolved content was centrifuged for 5 minutes at 10000 rpm.

Equipment (HPLC System)

Instrument:	Agilent/ 1200 Series
Detector:	DAD
Column:	Agilent, Zorbax SB-C18 5 µm, 4.6 x 150 mm
Mobile phase:	Isocratic mode: A: 0.1% trifluoroacetic acid in water (60%) B: acetonitrile (40%)
Flow rate:	0.6 mL/min
Column temp.:	35 °C
Wavelength:	220 nm
Injection volume:	10 µL
Run time:	10 minutes
Retention time:	~3.34 min.

Results and discussions

Table A 117: Summary of recovery experiments

Dose Level	Sample	Final concentration (mg/mL)	% Recovery	Mean % recovery	SD	% RSD
Control Feed	1	0.000	0.00	0.00	0.00	0.00
Low Dose	1	0.116	112.62	112..62	0.00	0.00
	2	0.116	112.62			
	3	0.116	112.62			
	4	0.116	112.62			
	5	0.116	112.62			
High Dose	1	1.074	107.40	106.94	0.38	0.36
	2	1.072	107.09			
	3	1.067	106.59			
	4	1.064	106.51			
	5	1.071	107.10			
Control Feed	1	0.000	0.00	0.00	0.00	0.00
High Dose 1	1	1.136	113.37	113.24	0.45	0.40
	2	1.142	113.97			
	3	1.133	112.96			
	4	1.134	112.95			
	5	1.134	112.95			

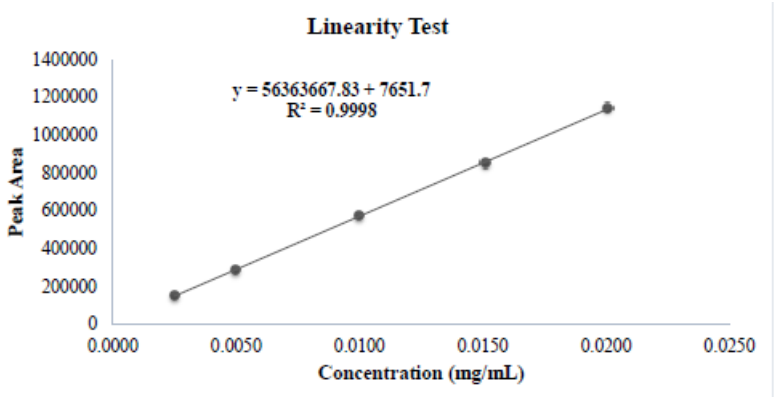
Accuracy and precision / repeatability

The results for accuracy and precision showed RSD values of < 10% for each spiking level and overall and recovery values within the required range of 70—110%. Two untreated samples were analysed with recovery values < LOD.

Linearity

Linearity was determined by 5-points calibration curve (single injections) using matrix matched standards at a range of 0.0025 – 0.0201 mg/mL ($R^2 > 0.99$). The standard solutions were injected in single replications for each level.

ID	Concentration (mg/mL)	Peak Area
Linearity level-1	0.0025	150367
Linearity level-2	0.0050	288144
Linearity level-3	0.0100	574553
Linearity level-4	0.0151	849796
Linearity level-5	0.0201	1145764



Limit of quantification

Based on the available data, the LOQ was estimated to be 0.005 mg/mL.

Limit of detection

Based on the available data, the LOD was estimated to be 0.0025 mg/mL.

Matrix effects

Matrix-matched standard solutions were used.

Specificity

The method was regarded specific with a stable retention time of the analyte of 3.34 min. No interference of the matrix was observed at the retention time of analyte (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

No information given. However, high recoveries demonstrated the storage stability of the samples and sample extracts.

Table A 118: Characteristics of the analytical method validation for the determination of RH-141455 in rat feed

	RH-141455
Specificity	Typical chromatograms provided. Stable retention time of the analyte of 3.34 min. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 5-point calibration, linear. y = 56363667.83 + 7651.7 Correlation coefficient r^2 was >0.99 Individual calibration data and calibration line equation presented in the study report
Calibration range	0.0025 mg/mL – 0.0201 mg/mL
Assessment of matrix effects is presented	Yes.
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ estimated to be 0.005 mg/mL LOD was estimated to be 0.0025 mg/mL

The following figure shows a typical chromatogram.

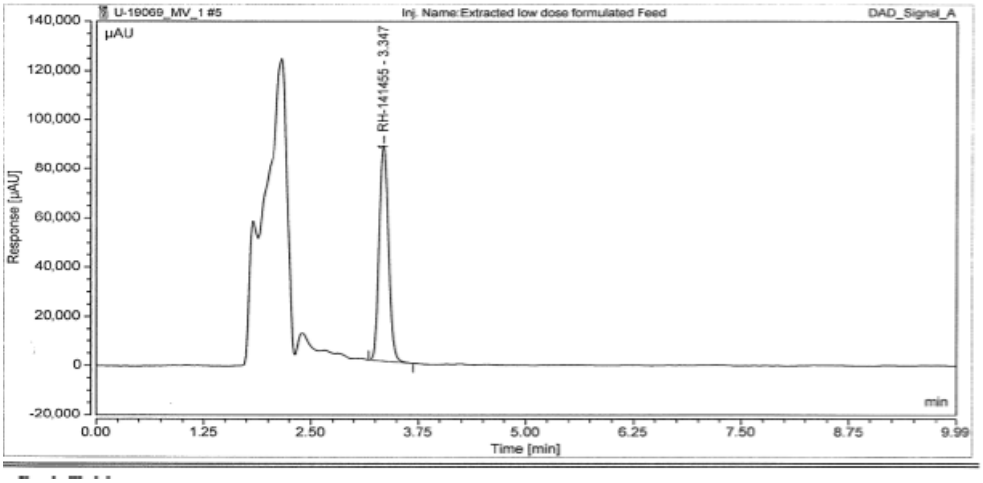


Figure A 55: **Typical Chromatogram of RH-141455**

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-141455 in rat feed.

(xxx 2019)

A 2.1.1.10 Description of analytical method for the determination of residues in bees' feed items

A 2.1.1.10.1 Analytical method 1

A 2.1.1.10.1.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.1/74**
Report	Picard, Ch. R., 2018: Zoxamide: Honey bee (*Apis mellifera* L.) larval toxicity, repeated exposure Exigent LLC, A Gowan Group Company, USA Smithers Viscient, USA, Report No. 12791.6307, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

An analytical method for the determination of zoxamide present in royal jelly diet of honey bees has been validated according to SANCO/3029 rev. 4 (2000). Recovery samples were initially diluted with 80/20 acetone/purified reagent water (v/v) and were subsequently diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). Recovery samples were analysed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Equipment

LC-MS/MS System:	MDS Sciex API 5000 mass spectrometer
Column:	Phenomenex Kinetex C18, 2.6 µm, 2.1 × 50 mm
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile

Gradient:

Time [min]	Solvent A [%]	Solvent B [%]
0.50	95.0	5.00
2.50	0.00	100
3.90	0.00	100
4.00	95.0	5.00
5.00	95.0	5.00

Flow rate:	0.600 mL/min
Column temp.:	40°C
Injection volume:	100 µL
Run Time	5.00 minutes
Retention time:	Approx. 2.7 min
Ionization Mode:	Positive (+) ESI
Scan type:	MRM
Q1/Q3 Masses:	336.094/187.20 amu

For further MS conditions, please refer to the study report.

Results and discussions

Table A 119: Summary of recovery experiments

Sample ID: 12791-6308-	Sample Type	Fortified Concentration (µg a.i./g)	Retention Time (minutes)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
14	Reagent Blank	0.00	NA[a]	5000	<0.250[b]	NA
15	Control	0.00	NA	5000	<0.250	NA
16	Control	0.00	NA	5000	<0.250	NA
17	LOQ	0.500	2.73	5000	0.542	108
18	LOQ	0.500	2.74	5000	0.532	106
19	LOQ	0.500	2.74	5000	0.515	103
20	LOQ	0.500	2.74	5000	0.513	103
21	LOQ	0.500	2.74	5000	0.514	103
		Mean:	**2.74**		**0.523**	**105**
		SD[c]:	**0.00447**		**0.0131**	**2.63**
		% RSD[d]:	**0.163**		**2.51**	**2.51**
22	High	5000	2.74	25.000.000	5200	104
23	High	5000	2.74	25.000.000	5130	103
24	High	5000	2.73	25.000.000	5440	109
25	High	5000	2.73	25.000.000	5240	105
26	High	5000	2.74	25.000.000	5120	102
		Mean:	**2.74**		**5230**	**105**
		SD:	**0.00548**		**130**	**2.60**
		% RSD:	**0.200**		**2.49**	**2.49**
		Overall Mean:	**2.74**		**Overall Mean:**	**105**

	Overall SD:	**0.00483**		**Overall SD:**	**2.47**
	% RSD:	**0.176**		**% RSD:**	**2.36**
				N[e]:	**10**

[a] NA = Not Applicable
[b] Concentrations expressed as less than values were below the method detection limit (MDL). The MDL is dependent upon the lowest concentration calibration standard and the dilution factor of the controls (i.e., 0.0500 µg a.i./L × 5000 mL/g = 0.250 µg a.i./g).
[c] SD = Standard Deviation
[d] RSD = Relative Standard Deviation
[e] N = Total number of samples used to determine the overall mean, standard deviation (SD), and coefficient of variation (RSD)
NOTE: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Accuracy and precision / repaeatability

Recoveries ranged from LOQ (0.500 µg/g) with 105 ± 2.63% (RSD: 2.51%) to the highest successfully validated dose (5000 µg/g) with 105 ± 2.60% (RSD: 2.49%).

The results for accuracy and precision showed RSD values < 10 %.

Recovery values were within the required range of 70-110 % (i.e. 105 ± 2.36%).

Repeatability with 5 replicates for each level showed RSD values < 20% per level.

Linearity

Linearity was checked by a 6-points calibration curve (single injections) using matrix matched standard solutions at a range of 0.500 and 5000 µg a.i./g ($r^2 > 0.99$).

Limit of quantification

The limit of quantification was 0.500 µg a.i./g, the lowest fortification level.

Limit of detection

The method detection limit (MDL) was 0.250 µg a.i./g.

Matrix effects

Matrix-matched standard solutions were used.

Specificity:

The LC-MS/MS method was regarded specific for the analyte. No significant interference was observed at the retention time of zoxamide in blank standard samples (i.e. < 30% of the analyte peak area).

Storage stability

The concentration of zoxamide in refrigerated royal jelly diet remained stable for at least four days without degradation of the active substance, which allows the use of the same treated diets for three days.

Table A 120: Characteristics of the analytical method for the determination of zoxamide in royal gelee

	Zoxamide
Specificity	HPLC-MS/MS method. Mass spectra provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	6-point calibration with external matrix-matched standard. Linear. Calibration curve equation: $y = 155262.7229\ x - 249.6153$, $r^2 = 0.999$

	Zoxamide
Calibration range	0.500 and 5000 µg a.i./g
Assessment of matrix effects is presented	No. Matrix-matched standard solutions were used.
Limit of determination/quantification	LOQ: 0.500 µg a.i./g. LOD: 0.250 µg a.i./g

The following figure shows typical chromatogram.

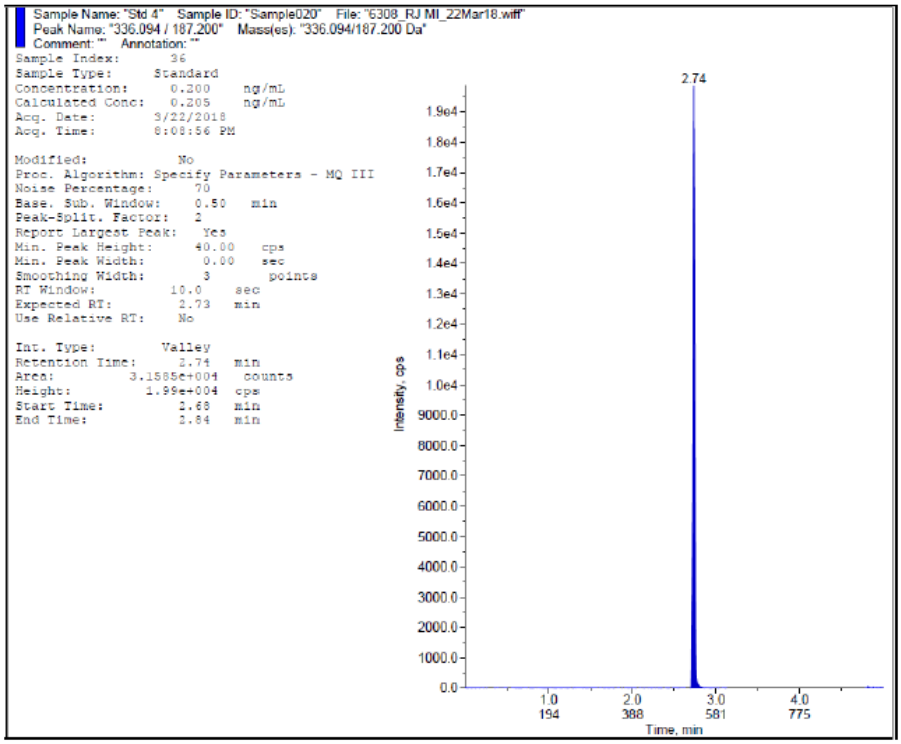


Figure A 56: Chromatogram of a calibration standard

Conclusion

The method for the determination of zoxamide in royal gelee diet was regarded valid according to SANCO/3029/99 rev. 4 (2000).

(Picard Ch. R. 2018)

A 2.1.1.10.2 Analytical method 2

A 2.1.1.10.2.1 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/75**
Report	Ruhland, S., 2018: Chronic toxicity of Cymoxanil 33% + Zoxamide 33% WG to the honey bee *Apis mellifera* L. under laboratory conditions Gowan Crop Protection Ltd., UK, Oxon Italia S.p.A, Italy

	BioChem agrar, Germany, Report No. 17 48 BAC 0005, 17 35 CRB 0009, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the verification of the concentration of the active ingredients cymoxanil and zoxamide in the test item feeding solutions. The determination was conducted by an in-house developed method using high performance liquid chromatography (HPLC) with UV-detection.

Determination was performed by high pressure liquid chromatography (HPLC) with UV-detection (UV). The specificity of the method was assured by continuously recorded UV spectra from 200 to 300 nm with a diode-array detector (DAD). Spectra of the test item peaks were compared to those of the references. As a result, similar spectra with approximately equal absorption maxima, constant chromatographic retention times and no interfering peaks were observed.

The method fulfils all criteria of the guidance document SANCO/3029/99 for the determination of both active substances:

– blank values do not exceed 30% of the lowest validated concentration,
– mean recoveries for each level are in the range 70-110%,
– the RSD is < 20% per level.

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 46.40 mg/L of cymoxanil and 46.69 mg/L of zoxamide.

No confirmatory method or ILV is required, since the method is used for pre-registration purposes only. The method was validated specifically for the present study.

Equipment

Instrument:	Shimadzu HPLC system with a triple quadrupole mass spectrometric detector
Column:	Macherey Nagel Nucleoshell RP18, 2.0 mm x 100 mm, 2.7 µm
Mobile phase:	A: Water with 0.1% (v/v) acetic acid + 10% (v/v) B B: Acetonitrile with 33% (v/v) MeOH and 0.1% (v/v) acetic acid

Gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0.00 min	95	5
8.00 min	5	95
10.00 min	5	95
10.01 min	95	5
12.00 min	Stop	Stop

Flow rate:	0.35 mL/min
Detection:	UV-detection at 240 nm for cymoxanil UV-detection at 211 nm for zoxamide
Retention time:	Approx. 3.5 min for cymoxanil Approx. 7.5 min for zoxamide

Results and discussions

Recovery findings

Table A 121: Recovery results from method validation of analytes using the analytical method

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Test medium*	Cymoxanil	46.40	97	0.2	None
Test medium*	Cymoxanil	1542	98	0.6	None
Test medium*	Zoxamide	46.69	88	0.6	None
Test medium*	Zoxamide	1551	85	6.3	None

* 50% (w/v) sucrose solution containing 0.1% (w/v) xanthan

Accuracyand precision / repeatability

Accuracy was tested by spiking sample matrix with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%.

Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level.

Linearity

Linearity was tested for 5 points at a concentration range of at least ± 20% of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 46.40 mg/L of cymoxanil and 46.69 mg/L of zoxamide.

Matrix effects

Recoveries of validation samples were within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.

Specificity

The specificity of the method was assured by the following method: UV spectra from 200 to 300 nm were continuously recorded by the diode-array detector. Spectra of the peaks were compared to those of the reference. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.

Stability of sample extracts

The maximum storage time of deep-frozen feeding solutions was < 30 days. Extracts for analysis were stored for < 24 hours in the refrigerator.

Table A 122: Characteristics for the analytical method used for validation zoxamide and cymoxanil residues in bees

	Zoxamide	Cymoxanil
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks
Calibration (type, number of data points)	5-point calibration with external standard Linear calibration, no weighting was used.	5-point calibration with external standard Linear calibration, no weighting was used.

	Zoxamide	Cymoxanil
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks
	Calibration curve equation: y = 114408 x – 8153.70, r^2 = 0.9999827	Calibration curve equation: y = 45044.9 x – 2379.70, r^2 = 0.9999935
Calibration range	4.555 to 22.77 mg/L in analytical samples	4.344 to 21.72 mg/L in analytical samples
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 46.69 mg/L (regarding dilution factor 7.470 mg/L) LOD:	46.40 mg/L (regarding dilution factor 7.425 mg/L) LOD:

The following figure shows typical chromatogram.

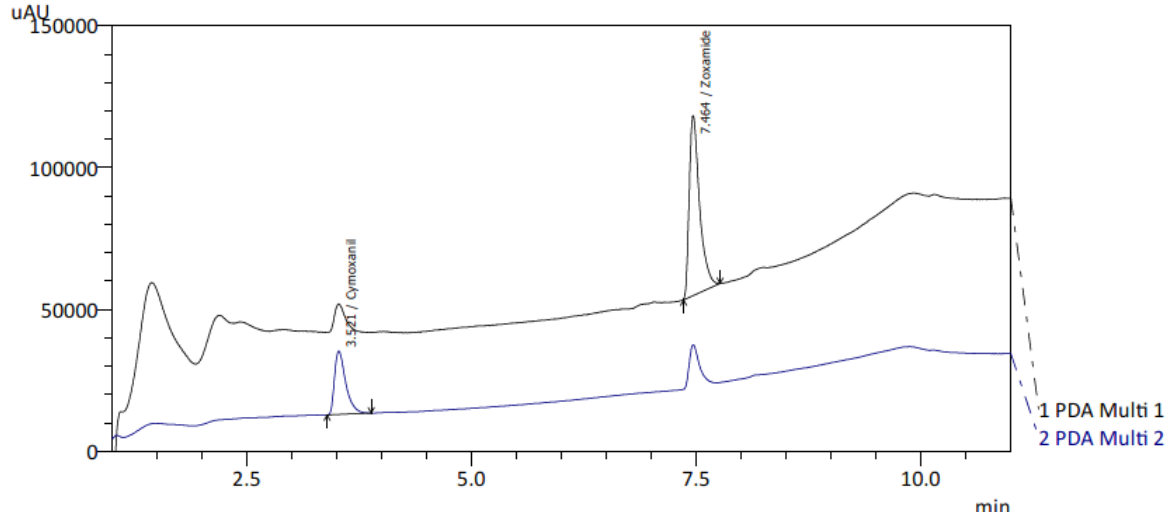


Figure A 57: **Chromatogram of cymoxanil and zoxamide**

Conclusion

A method for pre-registration purposes was successfully validated according to SANCO/3029/99 rev. 4 and regarded acceptable for the determination of zoxamide and cymoxanil in the test item feeding solutions. The nominal test item concentrations in the feeding solutions were analytically verified.

(Ruhland S. 2018)

A 2.1.1.10.3 Analytical method 3

A 2.1.1.10.3.1 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference: **KCP 5.1/76**

Report: Scheller, K., 2020: Cymoxanil 33% + Zoxamide 33% WG - Repeated exposure of honey bee (*Apis mellifera* L.) larvae under laboratory conditions (*in vitro*) Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 17 48 BLC 0005, 17 35 CRB 0010, GLP, Not published

Guideline(s): SANCO/3029/99 rev. 4 (2000)

Deviations: No

Acceptability: Yes

Materials and methods

The purpose of the analytical phase of the study was the verification of the concentration of the active ingredients cymoxanil and zoxamide in the test item stock solutions. The analytical method was validated according to SANCO/3029/99 rev. 4.

Determination was performed by high pressure liquid chromatography (HPLC) with UV-detection (UV). The specificity of the method was assured by continuously recorded UV spectra from 200 to 300 nm with a diode-array detector (DAD). Spectra of the test item peaks were compared to those of the reference items. As a result, similar spectra with approximately equal absorption maxima, constant chromatographic retention times and no interfering peaks were observed.

No confirmatory method is required, since the method is used for pre-registration purposes only. The method was validated specifically for the present study.

The recoveries of cymoxanil in the specimens were between 100% and 105%, the recoveries of zoxamide were between 87% and 91%. No active ingredient was detected in the control specimens. Thus, the test item concentrations in the stock solutions of the feeding solutions from the biological part were analytical verified.

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 8.646 mg/L of cymoxanil and 8.594 mg/L of zoxamide.

Equipment:

A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used.

Column: Macherey Nagel Nucleoshell RP18, 2.0 mm x 100 mm, 2.7 µm

Mobile phase: A: Water with 0.1% (v/v) acetic acid + 10% (v/v) B
B: Acetonitrile with 33% (v/v) MeOH and 0.1% (v/v) acetic acid

Gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0.00 min	95	5
8.00 min	5	95
10.00 min	5	95
10.01 min	95	5
12.00 min	Stop	Stop

Flow rate: 0.35 mL/min

Detection: UV-detection at 240 nm for cymoxanil

UV-detection at 211 nm for zoxamide

Retention time: Approx. 3.0 min for cymoxanil
Approx. 7.1 min for zoxamide

Results and discussions

Recovery findings

Table A 123: Recovery results

Matrix	Analyte	Fortification level (mg/L) (n=5)	Mean recovery (%)	RSD (%)	Comments
Test medium*	Cymoxanil	8.594	95	3.3	None
Test medium*	Cymoxanil	306.7	102	1.0	
Test medium*	Zoxamide	8.646	97	2.1	
Test medium*	Zoxamide	308.6	84	0.6	

* ASS containing 18% (w/v) glucose, 18% (w/v) fructose, 4% (w/v) yeast extract

Accuracyand precision / repeatability

Accuracy was tested by spiking sample matrix with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%. Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level.

Linearity

Linearity was tested for 5 points at a concentration range of at least ± 20% of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 8.646 mg/L of cymoxanil and 8.594 mg/L of zoxamide.

Matrix effects

Recoveries of validation samples were within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.

Specificity

The specificity of the method was assured by the following method: UV spectra from 200 to 300 nm were continuously recorded by the diode-array detector. Spectra of the peaks were compared to those of the reference. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.

Stability of sample extracts

The specimens for analysis were stored at ≤ -18 °C. The largest specimen storage was 16 days (≤ 30 days); therefore, storage stability testing was not necessary.

Table A 124: Characteristics for the analytical method used for validation zoxamide residues in bee larvae

	Cymoxanil	Zoxamide
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time of the analyte, no interfering peaks	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time of the analyte, no interfering peaks
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data presented in section 8.1. The calibration was linear, no weighting was used. Calibration curve equation: $y = 74965.2 x + 3179.15$, $r^2 = 0.9999753$	5-point calibration with external standard Individual calibration data presented in section 8.1. The calibration was linear, no weighting was used. Calibration curve equation: $y = 191327 x – 11410.4$, $r^2 = 0.9999883$
Calibration range	1.690 to 8.448 mg/L in analytical samples	1.847 to 9.233 mg/L in analytical samples
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
Limit of quantification (LOQ) Limit of detection (LOD)	8.646 mg/L (2.767 mg/L if the sample dilution is taken into account)	8.594 mg/L (2.750 mg/L if the sample dilution is taken into account)

The following figure shows a typical chromatogram.

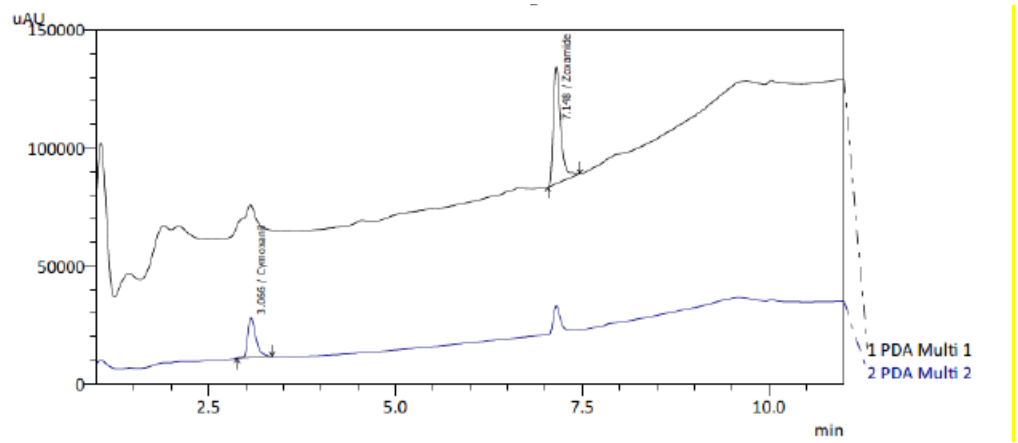


Figure A 58: Chromatogram of cymoxanil and zoxamide

Conclusion

A method for pre-registration purposes was successfully validated according to SANCO/3029/99 rev. 4 and regarded acceptable for the determination of zoxamide and cymoxanil in the test item feeding solutions. The nominal test item concentrations in the feeding solutions were analytically verified.

(Scheller K. 2020)

A 2.1.1.10.4 Analytical method 4 – determination of zoxamide and cymoxanil in bee feed

items

In the following studies the active substances zoxamide and cymoxanil have been analysed in spray solutions, nectar (forager bees and in-hive), bees (dead and alive bees), pollen (trap and in-hive) and flowers by a method following QuEChERS extraction inclusive clean-up as recommended by Anastassiades *et al*. (2003) with HPLC-MS/MS detection.

A 2.1.1.10.4.1 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/77**
Report	Schnurr, A., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 18 48 BFB 0001, 18 35 CRB 0040, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4 (2000) SANCO/825/00 rev. 8.1 (2010)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of cymoxanil and zoxamide after field application of the test item Cymoxanil 33% + Zoxamide 33% WG.

The analysis of specimens (spray solutions, nectar (forager bees and in-hive), bees (dead and alive bees), pollen (trap and in-hive) and flowers was conducted by an in-house development method based on the QuEChERS extraction method inclusive clean-up of Anastassiades *et al*. (2003) using high performance liquid chromatography (HPLC) with mass-spectrometric (MS-MS) detection of cymoxanil and zoxamide based on the methods of Jooß (2013) and Melkebeke (2000), respectively. However, the new method allows the detection of both active substances in one run.

The method involves the extraction of 1g samples (flowers and bees) with 2.5 mL acetonitrile and 2.5 mL water, followed by liquid/liquid partitioning after addition of 2g anhydrous MgSO4/NaCL (4:1 w/w). In case of the matrix pollen, extraction was done without 2.5 mL water. The sample extracts were cleaned by solid-phase extraction with 50 mg Envi-Carb (GCB), magnesium sulfate, PSA and C-18 (1:1:1:1, w/w/w/w). The sample extracts were diluted with acetonitrile/water/blank extract (25/50/25 v/v/v) containing 0.1% formic acid in a way that the concentration of the diluted extract was within the range of the respective calibration curve. The active substances in the cleaned extracts were separated on a reverse phase column by high performance liquid chromatography (HPLC). They were detected by tandem mass-spectrometry monitoring two mass transitions each (cymoxanil: m/z 199 →128 and 199 → 111), zoxamide: m/z 336 → 187 and 336 → 159) for quantification and/or qualification, respectively. The analysis was performed with external standard solutions (for spray solutions) or matrix-matched standards (for flowers, nectar, bees and pollen). The limit of quantification (LOQ) of the method was set at 0.005 mg/L (for spray solution) and at 0.005 mg/kg (for solid commodity) per analyte. The method was fully validated according to SANC0/3029/99 rev. 4 taking into account additional measures as required in SANC0/825/00 (2010).

Equipment

HPLC system: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector was used

Column: ACE Excel 3 C18-AR, 3µm, 100*2.1 mm

Mobile phase: A: Water containing 0.1% formic acid
B: Methanol containing 0.1% formic acid

Gradient:

Time (min)	A (%)	B (%)
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25
9.00	Stop	Stop

Flow rate: 0.4 mL/min

Run time 9.00 min

Oven Temperature: 40°C

Injection volume: 20µL

Retention time: 3.2 min for cymoxanil
5.4 min for zoxamide

Detection: ESI positive, MRM,
Cymoxanil: m/z 199.10 → 128.10 (quantifier), 199.10 → 111.00 (second quantifier of qualifier)
Zoxamide: m/z: 336.00 → 186.95 (quantifier), 336.00 → 159.00 (second quantifier of qualifier)

Results and discussions

Recovery findings

Table A 125: Recovery results from method validation of the quantifier

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil 199.10 → 128.10 (quantifier)		Zoxamide 336.00 → 186.95 (quantifier)	
				Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	LOQ	5	0.005	102	4-3	92	14.5
	200 x LOQ	5	1.000	97	2-9	89	5.5
	Blank	2	0.000	n.d.	-	n.d.	-
Nectar	LOQ	5/4*	0.005	80	9.9	71	4.2
	200 x LOQ	5	1.000	97	1.6	91	5.3
	Blank	2	0.000	n.d.	-	n.d.	-
Bees	LOQ	4**	0.005	103	3.0	84	6.5
	200 x LOQ	5	1.000	99	1.2	76	1.5
	Blank	2	0.000	n.d.	-	n.d.	-
Pollen	LOQ	4**	0.005	105	12.3	109	7.8
	200 x LOQ	5	1.000	109	2.8	105	3.7
	Blank	2	0.000	n.d.	-	n.d.	-
Flowers	LOQ	5	0.005	109	7.6	100	13.2

	200 x LOQ	5	1.000	108	3.1	109	2.8
	Blank	2	0.000	n.d.	-	n.d.	-

* for cymoxanil 5 replicates were taken into account and for zoxamide 4 replicates were taken into account since one sample was an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
** one sample was not taken into account since it is an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
n.d. = not detected
n = replicates

Table A 126: Recovery results from method validation of the qualifier

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil 199.10 → 111.00 (qualifier)		Zoxamide 336.00 → 159.00 (qualifier)	
				Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	LOQ	5	0.005	104	6.7	96	15.4
	200 x LOQ	5	1.000	98	1.9	89	5.9
	Blank	2	0.000	n.d.	-	n.d.	-
Nectar	LOQ	5/4*	0.005	75	10.7	74	4.6
	200 x LOQ	5	1.000	97	2.0	91	4.2
	Blank	2	0.000	n.d.	-	n.d.	-
Bees	LOQ	4**	0.005	81	16.7	76	7.0
	200 x LOQ	5	1.000	86	6.3	75	1.7
	Blank	2	0.000	n.d.	-	n.d.	-
Pollen	LOQ	4**	0.005	88	13.4	101	13.6
	200 x LOQ	5	1.000	98	4.2	105	4.0
	Blank	2	0.000	n.d.	-	n.d.	-
Flowers	LOQ	5	0.005	84	16.4	94	13.1
	200 x LOQ	5	1.000	107	6.7	109	4.4
	Blank	2	0.000	n.d.	-	n.d.	-

* for cymoxanil 5 replicates were taken into account and for zoxamide 4 replicates were taken into account since one sample was an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
** one sample was not taken into account since it is an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
n.d. = not detected
n = replicates

Accuracyand precision / repeatability

The recovery values were in the range of 70-110% with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for solvent calibration and all matrix matched calibration curves (6 concentration levels) of cymoxanil in the range of 0.101 – 10.10 µg/L (corresponding to 0.001 – 2.02 mg/kg for solid commodities) and zoxamide in the range of 0.101 – 10.08 µg/L (corresponding to 0.001 – 2.02 mg/kg for solid commodities). This covers ranges from at least 20% of the LOQ to at least 202% above 200 x LOQ, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was 0.005 mg/L (for spray solution) and 0.005 mg/kg (for flowers, nectar, bees, pollen) of cymoxanil and zoxamide (corresponding to 0.499 µg/L of cymoxanil and 0.502 µg/L of zoxamide in diluted samples and samples extract).

Limit of detection

The limit of detection (LOD) was 0.001 mg/L (for spray solution) and 0.001 mg/kg (for flowers, nectar, bees, pollen) of cymoxanil and zoxamide (corresponding to 0.101 µg/L of cymoxanil in diluted samples and samples extract, corresponding to 0.101 µg/L of zoxamide in diluted samples and samples extract).

Matrix effects

Matrix effects > 20% were observed for flowers, nectar, bees and pollen samples. For pollen and bee extracts, a strong suppression with up to approximately 50% was demonstrated. The nectar extracts show a strong enhancement partially with over 300%. The flowers extracts show strong enhancement at low concentration and a slightly suppression at high concentrations. Therefore, matrix-matched standard solutions were used for the solid commodities.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

During the whole experimental phase of 11 days in fridge at 4-10°C, the samples were stable (difference of the mean recoveries: < 20% in 50% methanol containing 0.1% formic aid. The 11 days old storage samples were measured against fresh prepared samples.

Storage stability of frozen samples

Samples were stored for at max. 362 days in the freezer (at ≤ -18°C). Therefore, the freezer storage stability was tested with freshly spiked retain (control) samples analysed in parallel to stored samples and proofed acceptable for both active substances.

Table A 127: Storage stability

Matrix	Sample	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil		Zoxamide	
			Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	Stored	1.000	95	0.3	92	10.2
	Fresh	1.000	96	1.5	92	13.0
	Difference	-	1	-	0	-
Nectar	Stored	1.000	106	1.2	84	0.9
	Fresh	1.000	98	0.7	91	0.8
	Difference	-	8	-	7	-
Bees	Stored	1.000	87	1.4	72	1.5
	Fresh	1.000	97	0.6	78	1.6
	Difference	-	10	-	6	-
Pollen	Stored	1.000	80	0.7	106	13.9
	Fresh	1.000	98	2.1	105	1.0
	Difference	-	18	-	1	-

Flowers	Stored Fresh	1.000	98	11.9	96	16.7
		1.000	108	0.8	108	1.4
	Difference	-	10	-	12	-

Table A 128: Characteristics of the analytical method for spay solution

	Zoxamide	**Cymoxanil**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/solvent calibration. The calibration was linear and weighed 1/c. Calibration curve equation: y = 18331.4 x + 128.725, r^2 = 0.99948	6-point calibraqtion with external standard/solvent calibration. The calibration was linear and weighed 1/c. Calibration curve equation: y = 60463.2 x + 657.923, r^2 = 0.99976
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./L spray solution)*	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./L spray solution)*
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L*, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L*, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

* In case of spray solution: mg/kg = mg/L

Table A 129: Characteristics of the analytical method for nectar

	Zoxamide	**Cymoxanil**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	equation: y = 20251.5 x + 4572.22, r^2 = 0.99988	equation: y =57548.2 x + 12781.3, r^2 = 0.99980
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

Table A 130: Characteristics of the analytical method for bees

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (25% v/v extract). The calibration was linear and weighed 1/c. Calibration curve equation: y = 13715.0 x + 923.474, r^2 = 0.99997	6-point calibraqtion with external standard//matrix matched calibration (25% v/v extract). The calibration was linear and weighed 1/c. Calibration curve equation: y =50253.7 x + 4249.81, r^2 = 0.99983
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering	Recoveries of validation samples within 70-110%. No interfering

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

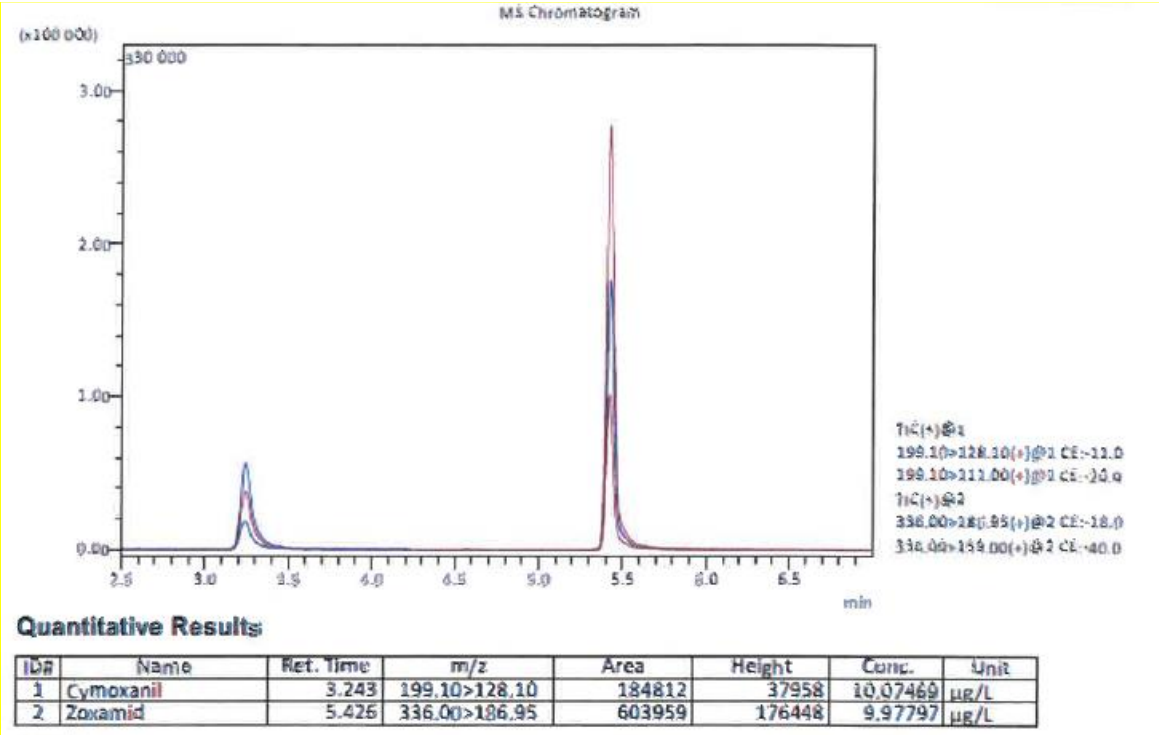
Table A 131: Characteristics of the analytical method for pollen

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 24181.7 x + 1556.20, r^2 = 0.99969	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y =43166.0 x + 2014.47, r^2 = 0.99938
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

Table A 132: Characteristics of the analytical method for flowers

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 20679.9 x + 6396.70, r^2 = 0.99955	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 34213.2 x + 12077.5, r^2 = 0.99996
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

The following figure shows a typical chromatogram.



ID#	Name	Ret. Time	m/z	Area	Height	Conc.	Unit
1	Cymoxanil	3.243	199.10>128.10	184812	37958	10.07469	µg/L
2	Zoxamid	5.426	336.00>186.95	603959	176448	9.97797	µg/L

Figure A 59: Chromatogram of cymoxanil and zoxamide

Conclusion

The method was fully validated on the test matrices spray solution, nectar, bees, pollen and flowers spiked with the test item (Cymoxanil 33% + Zoxamide 33% WG) at LOQ (0.005 mg/kg) and 200x LOQ (1 mg a.i./kg). As a result, all requirements of SANC0/3029/99 rev. 4 and SANC0/825/00 rev 8.1 were fulfilled

(Schnurr A. 2020)

A 2.1.1.10.4.2 Method validation

Comments of zRMS:	The method is acceptable. The validation parameters are consistent with the requirements.

Reference:	**KCP 5.1/78**
Report	Schnurr, A., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions in Spain (Southern zone) with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 19 48 BFB 0001, 18 35 CRB 0086, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANCO/825/00 rev. 8.1 (2010)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of cymoxanil and zoxamide after field application of the test item Cymoxanil 33% + Zoxamide 33% WG in spray solutions, nectar (forager bees and in-hive), flowers, pollen (trap and in-hive) and bees (dead and alive bees).

Sample preparation of flowers, pollen and bees was based on the QuEChERS extraction method including clean-up of extracts according to Anastassiades et al. (2003). The analyses were carried out using high performance liquid chromatography (HPLC) with mass-spectrometric (MS-MS) detection of cymoxanil and zoxamide based on the methods of Jooß (2013) and Melkebeke (2000). However, the method allows the detection of both active substances in one run.

Sample preparation involves the extraction of 1 g sample (flowers and bees) with 2.5 mL acetonitrile and 2.5 mL water, followed by liquid/liquid partitioning after addition of 2 g anhydrous MgSO4/NaCl (4:1 w/w). In case of the matrix pollen, extraction was done without 2.5 mL water. The sample extracts were cleaned up by dispersive solid-phase extraction using each 50 mg Envi-Carb (GCB), MgSO4, PSA and C-18 (1:1:1:1, w/w/w/w). The sample extracts were diluted with acetonitrile/water/blank extract (25/50/25 v/v/v) containing 0.1% formic acid in a way that the concentration of the diluted extract was within the range of the respective calibration curve. The active substances in the purified extracts were separated on a reversed-phase column by high performance liquid chromatography (HPLC). Detection was carried out by tandem mass-spectrometry, monitoring two mass transitions for each substance (cymoxanil: m/z 199 → 128 and 199 →111; zoxamide: m/z 336 → 187 and 336 → 159) for quantification and/or qualification, respectively. The analysis was performed with external standard solutions (for spray solutions) or matrix-matched external standards (for flowers, nectar, bees and pollen). The limit of quantification (LOQ) of the method was set at 0.005 mg/L (for spray solution) and at 0.005 mg/kg (for solid commodities) per analyte. The method was fully validated according to SANCO/3029/99 rev. 4 taking into account additional measures as required in SANCO/825/00 (2010) (Reference: Biochem project No.: 18 35 CRB 0040, analytical phase to Biochem project No.: 18 48 BFB 0001).

Equipment

Instrument: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector

Column: ACE Excel 3 C_{18}-AR, 3µm, 100*2.1 mm

Mobile phase: A: Water containing 0.1% formic acid
B: Methanol containing 0.1% formic acid

Gradient:

Time (min)	A (%)	B (%)
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25
9.00	Stop	Stop

Flow rate: 0.4 mL/min

Run time 9.00 min

Oven Temperature: 40°C

Injection volume: 20µL
40µL (bee specimens)

Retention time: Cymoxanil: 3.3 min
Zoxamide: 5.4 min

Detection: ESI positive, MRM,
Cymoxanil: m/z 199.10 → 128.10 (quantifier), 199.10 → 111.00 (second quantifier of qualifier)

Zoxamide: m/z: 336.00 → 186.95 (quantifier), 336.00 → 159.00 (second quantifier of qualifier)

Results and discussions

Recovery findings

Table A 133: Recovery results from method verification (quantifier)

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])		Cymoxanil 199.1 → 128.1 (quantifier)		Zoxamide 336.00 → 186.9 (quantifier)	
			Cymoxanil	Zoxamide	Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	High	5	767.71	771.46	103	2.0	107	10.4
	200x LOQ	3	1.075	1.080	104	2.4	98	1.6
	LOQ	3	0.005	0.005	96	1.1	91	2.4
	Blank	1	0.000	0.000	-	-	-	-
Nectar	200x LOQ	3	1.018	1.023	99	1.2	82	2.7
	LOQ	3	0.005	0.005	105	2.8	82	10.6
	Blank	1	0.000	0.000	-	-	-	-
Flowers	High	5	50.07	50.31	87	4.9	96	3.9
	LOQ	3	1.018	1.023	87	4.9	92	5.1
	200x LOQ	3	0.005	0.005	108	7.9	96	8.8
	Blank	1	0.000	0.000	-	-	-	-
Pollen	200x LOQ	3	1.018	1.023	102	2.6	99	1.0
	LOQ	3	0.005	0.005	106	1.2	76	1.3
	Blank	1	0.000	0.000	-	-	-	-
Bees	200x LOQ	3	1.018	1.023	79	7.8	78	4.5
	LOQ	3	0.005	0.005	99	4.3	110	8.3
	Blank	1	0.000	0.000	-	-	-	-

n = replicates

Table A 134: Recovery results from method verification (qualifier)

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])		Cymoxanil 199.10 → 111.0 (qualifier)		Zoxamide 336.00 →159.0 (qualifier)	
			Cymoxanil	Zoxamide	Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	High	5	767.71	771.46	102	1.8	105	9.2
	200x LOQ	3	1.075	1.080	103	1.0	102	2.8
	LOQ	3	0.005	0.005	95	3.2	89	3.0
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Nectar	200x LOQ	3	1.018	1.023	102	1.6	83	1.7
	LOQ	3	0.005	0.005	100	6.6	105	15.7
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Flowers	High	5	50.07	50.31	*	*	94	5.6
	LOQ	3	1.018	1.023	*	*	91	3.5
	200x LOQ	3	0.005	0.005	*	*	93	5.0
	Blank	1	0.000	0.000	*	*	n.d.	-
Pollen	200x LOQ	3	1.018	1.023	101	2.4	98	2.1

	LOQ	3	0.005	0.005	103	9.8	73	0.9
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Bees	200x LOQ	3	1.018	1.023	73	8.7	80	7.6
	LOQ	3	0.005	0.005	80	6.8	102	7.5
	Blank	1	0.000	0.000	n.d.	-	n.d.	-

n = replicates
* confirmatory transition could not be evaluated quantitatively due to matrix effects

Accuracyand precision / repeatability

The recovery values were in the range of 70-110% with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for solvent calibration and all matrix matched calibration curves (8 concentration levels) of cymoxanil in the range of 0.100 to 10.0 µg/L and in the range of 0.099 to 9.89 µg/L for zoxamide. This covers ranges from about 20% of the LOQ to about 200% above 200x LOQ, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was 0.005 mg/L (for spray solution, corresponding to 2.3 µg/L of cymoxanil and zoxamide in diluted samples) and 0.005 mg/kg (for nectar, flowers, pollen, bees) of cymoxanil and zoxamide (corresponding to 0.51 µg/L of cymoxanil and zoxamide in diluted samples and samples extracts).

Limit of detection

The limit of detection (LOD) was 0.001 mg/L (for spray solution) and 0.001 mg/kg (for nectar, flowers, pollen and bees) of cymoxanil and zoxamide.

Matrix effects

Matrix effects > 20% were observed for flowers, nectar, bees and pollen samples. For pollen and bee extracts, a strong suppression with up to approximately 50% was demonstrated. The nectar extracts show a strong enhancement partially with over 300%. The flowers extracts show strong enhancement at low concentration and a slightly suppression at high concentrations. Therefore, matrix-matched standard solutions were used for the solid commodities.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Stability of sample extracts was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. Sample extracts were stable for a period of 11 days at 4-10°C.The maximum storage period of sample extracts in this analytical phase was 6 days

Storage stability of frozen samples

For each matrix, freezer storage stability was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. The analytical results between stored and fresh samples

showed that the samples of spray solutions, nectar, bees, pollen and flowers are stable under frozen conditions for ≥ 1 year. The maximum storage period of specimens of study 19 48 BFB 0001 was 350 days at ≤-18°C

Table A 135: Storage stability

Matrix	Sample	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil		Zoxamide	
			Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	Stored	1.000	95	0.3	92	10.2
	Fresh	1.000	96	1.5	92	13.0
	Difference	-	1	-	0	-
Nectar	Stored	1.000	106	1.2	84	0.9
	Fresh	1.000	98	0.7	91	0.8
	Difference	-	8	-	7	-
Bees	Stored	1.000	87	1.4	72	1.5
	Fresh	1.000	97	0.6	78	1.6
	Difference	-	10	-	6	-
Pollen	Stored	1.000	80	0.7	106	13.9
	Fresh	1.000	98	2.1	105	1.0
	Difference	-	18	-	1	-
Flowers	Stored Fresh	1.000	98	11.9	96	16.7
		1.000	108	0.8	108	1.4
	Difference	-	10	-	12	-

Table A 136: Characteristics of the analytical method for spay solution

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c. Calibration curve equation: $y = 14885.5 x + 3451.72$, $r^2=0.99984$	8-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: $y = 22687.6 x + 8715.03$, $r^2=0.99986$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Limit of determination/ quantification	LOQ = 0.005 mg/L*, corresponding to 2.30 µg/L in the analytical sample. LOD = 0.001 mg/L*, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/L, corresponding to 2.31 µg/L in the analytical sample. LOD = 0.001 mg/L, corresponding to 0.099 µg/L in the analytical sample

Table A 137: Characteristics of the analytical method for nectar

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 6280.32 x + 2541.11$, $r^2=0.99927$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 37803.7 x + 32501.3$, $r^2=0.99945$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 138: Characteristics of the analytical method for flowers

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 21132.7 x + 519.863$, $r^2=0.99853$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 42436.9 x + 9228.50$, $r^2=0.99972$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 139: Characteristics of the analytical method for pollen

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 31441.2 x + 252.450$, $r^2=0.99979$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 47678.9 x + 4721.60$, $r^2=0.99997$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	peaks >30% of the lowest validation samples).	peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.458 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.460 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 140: Characteristics of the analytical method for bees

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 30101.0 x – 509.377$, $r^2=0.99987$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 16274.3 x + 366.132$, $r^2=0.99991$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

The following figures show a typical chromatogram.

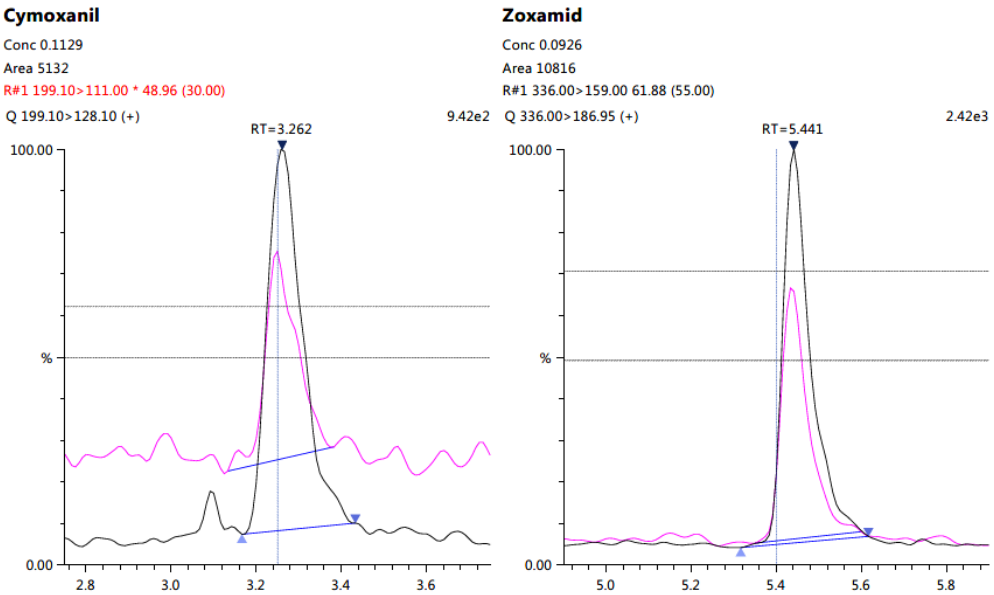


Figure A 60: Chromatogram of the lowest calibration standard (0.099 µg/L of cymoxanil (199.10 → 128.10 (quantifier)); 0.100 µg/L of zoxamide 336.00 → 186.95 (quantifier))

Conclusion

The method was verified on the test matrices spray solution, nectar, bees, pollen and flowers spiked with the test item (Cymoxanil 33% + Zoxamide 33% WG) at LOQ (0.005 mg/kg) and 200x LOQ (1 mg a.i./kg). For spray solutions and flowers, an additional validation level was analysed in order to cover the residues found in the original specimens. As a result, all requirements of SANCO/3029/99 rev. 4 and SANCO/825/00 rev 8.1 were fulfilled. For method validation details, please refer to the analytical phase report of BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001

(Schnurr A. 2020)

A 2.1.1.1 Description of analytical method for the determination of residues in non-target plants

A 2.1.2 Methods for post-authorisation 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)

A 2.1.2.1.1 Analytical method 1 – determination of RH-141455 and RH-141452 in root and tuber crops

EFSA (2017) has requested *"A fully validated monitoring method for RH-141455 and RH-141452 in potatoes (relevant for the representative uses in potato; submission date proposed by the applicant: unknown; see Section 1)."* This method (Witte, 2020; report no. 18G10186-01-VMPL) is provided.

The method of Witte (2020) can be seen as primary and/or ILV enforcement/monitoring method for the determination of zoxamide (R/S and sum) and RH-141452 and RH-141455 in all matrices (high water content, acidic, high oil content and dry) – especially for the determination of RH-141452 and RH-141455 in raw and processed commodities of root and tuber crops.

Component(s) analysed: RH-141455 and RH-141452				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Potato tubers Potato flakes Potato chips Pickled silverskin onions	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 Report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 Report no. 18G10186-01-VMPL
Component(s) analysed: R- and S-zoxamide and sum				
Potato tubers Potato flakes Potato chips Pickled silverskin Onion bulbs	Primary / ILV Confirmatory	0.005 mg/kg	LC-MS/MS	Witte, 2020 Report no. 18G10186-01-VMPL

A 2.1.2.1.1.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.2/01**
Report	Witte, A., 2020: Validation of an analytical method for the determination of residues of R/S-isomers of zoxamide and metabolites RH-141452 and RH-141455 in 4 different matrices: potato tubers (water containing matrix), potato flakes (dry matrix), potato chips (fat containing matrix) and pickled silverskin onions (acidic matrix) Gowan Crop Protection Ltd., UK

	CIP Pforzheim, Germany., Report No. 18G10186-01-VMPL, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) OECD ENV/JM/MONO (2017)17 series 72/39
Deviations:	None
Acceptability:	Yes

Materials and methods

The data presented in this report demonstrate that the method used in analogy to the multiresidue method QuEChERS (EN15662) permits the determination of residues of zoxamide isomers in matrices potato chips, potato flakes, pickled silverskin onions and potato tubers. Furthermore, the method used for zoxamide metabolites RH-141452 and RH-141455 was proven to be suitable for all four tested matrices for RH-141452 and RH-141455, but unsuitable for zoxamide (racemate) itself. The methods were validated in different plant tissues according to the guideline SANCO/825/00 rev. 8.1 (2010), i.e. in potato chips, potato flakes, pickled silverskin onions and potato tubers. The methods were proven to be specific, accurate and precise and a good repeatability and recovery was found in all four matrix groups for all analytes except for zoxamide (racemate).

Control specimens of each matrix were analysed in duplicate and fortified specimens were analysed in quintuple. Since two characteristic mass transitions were used to monitor each analyte, the method achieves a high level of specificity and no additional confirmation was necessary.

Equipment for zoxamide isomers

HPLC-MS/MS:	Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP
Column:	Phenomenex LUX® 5 µm cellulose-3 150 mm × 4.6 mm, 5.0 µm (Part No. 00F-4493-E0)
Mobile phase:	A: Water + 0.1 % formic acid B: Acetonitrile + 0.1 % formic acid

Time (min)	A (%)	B (%)
0.00	40	60
0.10	40	60
8.00	25	75
8.10	40	60
12.00	40	60

Flow rate:	1 mL/min
Column temp.:	40°C
Injection volume:	100µL
Retention time:	(R) – Zoxamide ~ 3.8 min (S) – Zoxamide ~ 4.3 min
Quantification:	Peak areas of the fragment ions, matrix-matched external standards
Ion mode:	Zoxamide: m/z 336 → 187; m/z 338 → 189

Equipment for zoxamide (racemate) and metabolites

HPLC-MS/MS:	Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP
Column:	Phenomenex SynergieTM 4 µm Polar – RP 80A 150 mm × 4.6 mm, 4.0 µm (Part No. 00F-4493-E0)
Mobile phase:	A: Water + 0.05 % Acetic acid B: Metahnol + 0.05 % Acetic acid

Time (min)	A (%)	B (%)
0.00	80	20
2.00	40	60
5.00	20	80
9.50	20	80
9.60	80	20
15.10	80	20

Flow rate: 1 mL/min
Column temp.: 40°C
Injection volume: 100µL
Retention time: Zoxamide (racemate) ~ 8.9 min
RH-141452 ~ 5.7 min
RH-141455 ~ 3.5 min
Quantification: Peak areas of the fragment ions, matrix-matched external standards
Ion mode: **Zoxamide**: m/z 336 → 187; m/z 338 → 189
RH-141452:
m/z 219 → 175; m/z 221 → 147 for matrices potato Flaked, potato tubers and pickled silverskin onions;
m/z 221 → 147; m/z 221 → 177 for matrix potato chips
RH-141455:
m/z 233 → 189; m/z 235 → 191 for matrices potato flakes, potato tubers and pickled silverskin onions;
m/z 235 → 191; m/z 233 → 109 for matrix potato chips

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Summaries of the results are presented in the following table.

Table A 141: Results of accuracy, recovery precision and repeatability ((R)-zoxamide)

Matrix	Fortification level [mg/kg]	Recoveries			No. of analyses	Overall recovery	
		Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
(R) - Zoxamide SRM 336 → 187 (quantification)							
Potato tubers	0.005	108, 114, 120, 102, 110	111	6.1	5	105	9.3
	0.05	96, 92, 109, 90, 108	99	9.0	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato tubers	0.005	110, 114, 118, 102, 110	111	5.4	5	106	9.2
	0.05	97, 92, 113, 90, 110	100	10.5	5		
(R) - Zoxamide SRM 336 → 187 (quantification)							

Potato flakes	0.005	100, 100, 94, 92, 98	97	3.8	5	102	6.1
	0.05	101, 110, 109, 105, 108	107	3.4	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato flakes	0.005	102, 98, 96, 94, 102	98	3.6	5	103	5.6
	0.05	103, 111, 110, 104, 108	107	3.3	5		
(R) - Zoxamide SRM 336 → 187 (quantification)							
Potato chips	0.005	100, 94, 100, 102, 100	99	3.1	5	105	6.4
	0.05	111, 109, 117, 108, 106	110	3.8	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato Chips	0.005	98, 94, 100, 102, 100	99	3.1	5	105	6.9
	0.05	111, 111, 117, 108, 110	111	3.0	5		
(R) - Zoxamide SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.005	104, 110, 116, 118, 108	111	5.2	5	103	9.8
	0.05	87, 100, 93, 96, 98	95	5.3	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Pickled silverskin onion	0.005	102, 108, 118, 118, 108	111	6.3	5	103	10.2
	0.05	86, 98, 93, 96, 98	94	5.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 142: Results of accuracy, recovery precision and repeatability ((S)-zoxamide)

Matrix	Fortification level [mg/kg]	Recoveries			No. of analyses	Overall recovery	
		Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato tubers	0.005	112, 112, 128, 102, 110	113	8.4	5	109	8.3
	0.05	107, 94, 113, 102, 112	106	7.4	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato tubers	0.005	112, 114, 128, 104, 110	114	7.8	5	110	8.4
	0.05	108, 93, 115, 102, 111	106	8.1	5		
(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato flakes	0.005	100, 104, 100, 94, 106	101	4.6	5	104	6.2

	0.05	98, 110, 116, 108, 105	107	6.2	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato flakes	0.005	100, 104, 104, 96, 106	102	3.9	5	104	5.5
	0.05	95, 108, 113, 108, 108	106	6.3	5		
(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato chips	0.005	92, 90, 92, 90, 92	91	1.2	5	95	5.4
	0.05	97, 105, 102, 97, 93	99	4.8	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato chips	0.005	94, 86, 98, 90, 94	92	4.9	5	96	5.9
	0.05	101, 105, 101, 97, 93	99	4.6	5		
(S) - Zoxamide SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.005	116, 114, 114, 114, 124	116	3.7	5	107	10.4
	0.05	91, 97, 103, 97, 96	97	4.4	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Pickled silverskin onions	0.005	114, 116, 118, 118, 128	119	4.5	5	108	11.3
	0.05	94, 99, 104, 96, 94	97	4.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 143: Results of accuracy, recovery precision and repeatability (zoxamide (racemate))

Matrix	Fortification level [mg/kg]	Recoveries			No. of analyses	Overall recovery	
		Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato tubers	0.01	-	-	-	5	-	-
	0.10	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato tubers	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato flakes	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		

Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato flakes	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato chips	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato chips	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Pickled silverskin onions	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 144: Results of accuracy, recovery precision and repeatability (RH-141452)

Matrix	Fortification level [mg/kg]	Recoveries			No. of analyses	Overall recovery	
		Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
RH-141452 SRM 219 → 175 (quantification)							
Potato tubers	0.01	99, 100, 104, 93, 106	100	5.0	5	97	6.1
	0.1	98, 88, 92, 90, 99	93	5.2	5		
RH-141452 SRM 221 → 147 (confirmation)							
Potato tubers	0.01	103, 104, 108, 101, 101	103	2.8	5	99	6.9
	0.1	97, 90, 92, 87, 102	94	6.3	5		
RH-141452 SRM 219 → 175 (quantification)							
Potato flakes	0.01	114, 101, 92, 103, 115	105	9.2	5	100	8.6
	0.1	101, 97, 98, 91, 91	96	4.6	5		
RH-141452 SRM 221 → 147 (confirmation)							

Potato flakes	0.01	115, 110, 91, 104, 116	107	9.6	5	101	9.6
	0.1	100, 94, 99, 89, 95	95	4.6	5		
RH-141452 SRM 221 → 147 (quantification)							
Potato chips	0.01	111, 116, 95, 102, 113	107	8.1	5	108	6.3
	0.1	113, 99, 108, 108, 110	108	4.8	5		
RH-141452 SRM 221 → 177 (confirmation)							
Potato chips	0.01	114, 113, 97, 112, 106	108	6.6	5	108	6.0
	0.1	113, 97, 109, 112, 111	108	6.1	5		
RH-141452 SRM 219 → 175 (quantification)							
Pickled silverskin onions	0.01	102, 97, 95, 103, 102	100	3.6	5	99	4.4
	0.1	105, 91, 96, 96, 98	97	5.2	5		
RH-141452 SRM 221 → 147 (confirmation)							
Pickled silverskin onions	0.01	94, 90, 93, 101, 101	96	5.2	5	96	5.5
	0.1	106, 89, 97, 97, 95	97	6.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 145: Results of accuracy, recovery precision and repeatability (RH-141455)

Matrix	Fortification level [mg/kg]	Recoveries			No. of analyses	Overall recovery	
		Single values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
RH-141455 SRM 235 → 191 (quantification)							
Potato tubers	0.01	101, 100, 90, 91, 105	97	6.8	5	96	6.2
	0.1	98, 90, 96, 88, 102	95	6.1	5		
RH-141455 SRM 223 → 109 (confirmation)							
Potato tubers	0.01	105, 101, 106, 105, 104	104	1.8	5	101	5.2
	0.1	100, 93, 100, 91, 105	98	5.8	5		
RH-141455 SRM 233 → 189 (quantification)							
Fehler! Textmarke nicht definiert. Potato flakes	0.01	104, 94, 97, 85, 89	94	7.8	5	92	8.0
	0.1	93, 92, 101, 79, 89	91	8.7	5		
RH-141455 SRM 235 → 191 (confirmation)							
Potato flakes	0.01	103, 102, 96, 93, 100	99	4.2	5	95	6.8

	0.1	92, 93, 101, 82, 91	92	7.3	5		
RH-141455 SRM 223 → 189 (quantification)							
Potato chips	0.01	109, 110, 103, 105, 115	108	4.3	5	106	5.5
	0.1	108, 95, 111, 106, 100	104	6.2	5		
RH-141455 SRM 235 → 191 (confirmation)							
Potato chips	0.01	128, 104, 112, 120, 116	116	7.7	5	110	8.6
	0.1	113, 96, 106, 105, 102	104	6.0	5		
RH-141455 SRM 233 → 189 (quantification)							
Pickled silverskin onions	0.01	102, 92, 104, 101, 102	100	4.7	5	96	6.3
	0.1	96, 86, 91, 92, 93	92	4.0	5		
RH-141455 SRM 235 → 191 (confirmation)							
Pickled silverskin onions	0.01	102, 94, 99, 104, 100	100	3.8	5	96	5.7
	0.1	97, 86, 92, 93, 92	92	4.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Mean recovery values obtained by HPLC-MS/MS for all matrices comply with the standard acceptance criteria of guideline SANCO/825/00 rev. 8.1 (2010), which require that the mean recoveries should be within the range of 70 - 120%.

Excluding from this is zoxamide (racemate), which was not suitable for the extraction method like its metabolites RH-141452 and RH-141455.

Accuracy and precision / repeatability

Fortification experiments for zoxamide isomers were performed in five-fold at the limit of quantification (0.005 mg/kg) and at 10-fold of the limit of quantification (0.05 mg/kg). For zoxamide (racemate) and its metabolites RH-141452 and RH-141455, fortifications were performed at the 0.01 mg/kg (LOQ) and 0.1 mg/kg (10-fold LOQ).

Mean recovery values obtained by HPLC-MS/MS for all matrices comply with the standard acceptance criteria of guideline SANCO/825/00 rev. 8.1 (2010), which require that the mean recoveries should be within the range of 70 - 120%. Therefore, it can be concluded that the methods are applicable on all matrices under investigation using HPLC with MS/MS detection. Excluding from this is zoxamide (racemate), which was not suitable for the extraction method like its metabolites RH-141452 and RH-141455.

Moreover, all corresponding relative standard deviations are below 20% as required by the guideline SANCO/825/00 rev. 8.1 (2010). This indicates that the method demonstrates good precision and repeatability for the different plant tissues at the validated levels. The repeatability of the method was assessed on the basis of the obtained relative standard deviations for each fortification level

Linearity

The linearity of the detector response for (R)- and (S)-zoxamide was confirmed by injecting eight matrix matched standard solutions (same solutions as described in 6.4) covering the working range of 0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions (eight-point calibration) and 0.0125 µg/L

to 10 µg/L for matrices potato flakes and potato chips (seven-point calibration) with correlation coefficients of $r \geq 0.998$.

The linearity of the detector response for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 was confirmed by injecting seven matrix matched standard solutions (same solutions as described in 6.4) covering the working range of 0.3 µg/L to 20 µg/L for all matrices with correlation coefficients of $r \geq 0.997$.

The lower margin of the linearity test was below 30% of the LOQ and the upper margin was higher by at least 20 % as the 10-fold LOQ. These margins cover the minimum range as required by SANCO/825/00 rev.8.1 (2010).

Limit of quantification

The limit of quantification (LOQ) was defined as the lowest fortification level at 0.005 mg/kg for zoxamide Isomers and 0.01 mg/kg for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 with mean recoveries ranging from 70% to 120% at a relative standard deviation (RSD) of ≤ 20% and blanks not exceeding 30% of the LOQ. These criteria were fulfilled for all matrices and analytes except for zoxamide (racemate), for which the tested method was deemed unsuitable.

Limit of detection

The limit of detection (LOD) was defined as 30% of the limit of quantification as required by SANCO/825/00 rev. 8.1 (2010) for residues in control samples (i.e. 0.0015 mg/kg and 0.003 mg/kg). Residues in the untreated samples used for recovery experiments were below 30% of the LOQ, below the limit of detection (LOD) to be exact.

Matrix effects

The matrix effect was tested at concentrations levels of 10µg/L for all matrices. Standard solutions were injected in pure solvent (double injection) against matrix-matched standard solutions.

A considerable matrix effect of > 20 % for (S)–zoxamide was observed in potato tubers and pickled silverskin onions. No significant matrix effects (above 20 %) could be observed for (R)–zoxamide in any matrix. Nevertheless, matrix matched standards were used for all matrices.

For zoxamide (racemate), significant matrix effects of > 20 % were observed for all matrices except for pickled silverskin onions. Metabolites RH-141452 and RH-141455 showed significant matrix effects of > 20 % for all matrices.

Specificity

Analysis of control specimens (in duplicate) of each matrix with HPLC-MS/MS using two characteristic mass transitions yielded no residues of zoxamide (racemate) or its metabolites RH-141452 and RH-141455 above 30 % of the LOQ, indicating that no significant interferences were present.

Zoxamide parent compound with two characteristic mass transitions: m/z = 336 → 187 (quantification) and 338 → 189 (confirmation) for all matrices.

- RH-141452: 219 → 175 and 221 → 147 for matrices potato flakes, potato tubers and pickled silverskin onions.
- RH-141452: 221 → 147 and 221 → 177 for matrix potato chips.
- RH-141455: 233 → 189 and 235 → 191 for matrices potato flakes, potato chips and pickled silverskin onions.
- RH-141455: 235 → 191 and 233 → 109 for matrix potato tubers.

Stability of sample extracts

The stability of zoxamide isomers as well as zoxamide (racemate) and its metabolites RH-141452 and RH-141455 in final extracts was assessed by measuring matrix-matched calibration standards of each matrix stored refrigerated (≤ 8 °C) against freshly prepared matrix-matched calibration standards. Matrix-matched calibration standards were prepared in the final dilution of control samples. Stability tests for all analytes in final extracts were performed at a level of 10µg/L. The results demonstrate that no significant deviation (> 20 %) between stored and freshly prepared matrix-matched standards was observed for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 after storage in a refrigerator (≤ 8 °C) for 8 (potato tubers), 7 (pickled silverskin onions), 8 (potato flakes) and 6 days (potato chips). Furthermore, no significant deviation (> 20 %) between stored and freshly prepared matrix-matched standards was observed for zoxamide isomers during 6 days of storage for all matrices.

The stability of the working solutions SM-Z1 and SM-RH1 (concentration 1 mg/L used for preparation of recoveries and standards; stored at ≤ 8 °C) was tested for 86 days. For analysis, stored and freshly prepared working solutions were diluted with acetonitrile (for zoxamide isomers) or methanol (for zoxamide (racemate) and its metabolites RH-141452 and RH-141455) to receive 100 µg/L standard solutions. Each standard was injected twice. The results demonstrate that no significant degradation (> 20 %) of zoxamide and its metabolites occurred in the working solution during storage.

Table A 146: Characteristics for the analytical method used for validation of zoxamide isomers, zoxamide (racemate) and metabolites residues in plant matrices

	(R) – (S) - zoxamide	**Zoxamide (racemate), RH-141452 and RH-141455**
Specificity	Mass spectrum is provided. Blank value above 30 % LOQ	Mass spectrum is provided. Blank value above 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration. Calibration range 0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions (eight-point calibration) and 0.0125 µg/L to 10 µg/L for matrices potato flakes and potato chips (seven-point calibration). Correlation coefficient r^2 was >0.998 for each individual matrix. Individual calibration data and calibration line equation presented in the study report.	Standard solution and matrix matched calibration. Calibration range 0.3 µg/L to 20 µg/L for all matrices (seven-point calibration). Correlation coefficient r^2 was >0.997 for each individual matrix. Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions and 0.0125 µg/L to 10 µg/L for matrices potato flakes and potato chips	0.3 µg/L to 20 µg/L for all matrices
Assessment of matrix effects is presented	yes	Yes
Limit of determination/quantification	LOQ: 0.005 mg/kg of each zoxamide isomer LOD: 30 % of the LOQ (*i.e*. 0.0015 mg/kg)	LOQ: 0.01 mg/kg for each analyte LOD: 30 % of the LOQ (*i.e*. 0.003 mg/kg)

The following figures show representative mass spectra.

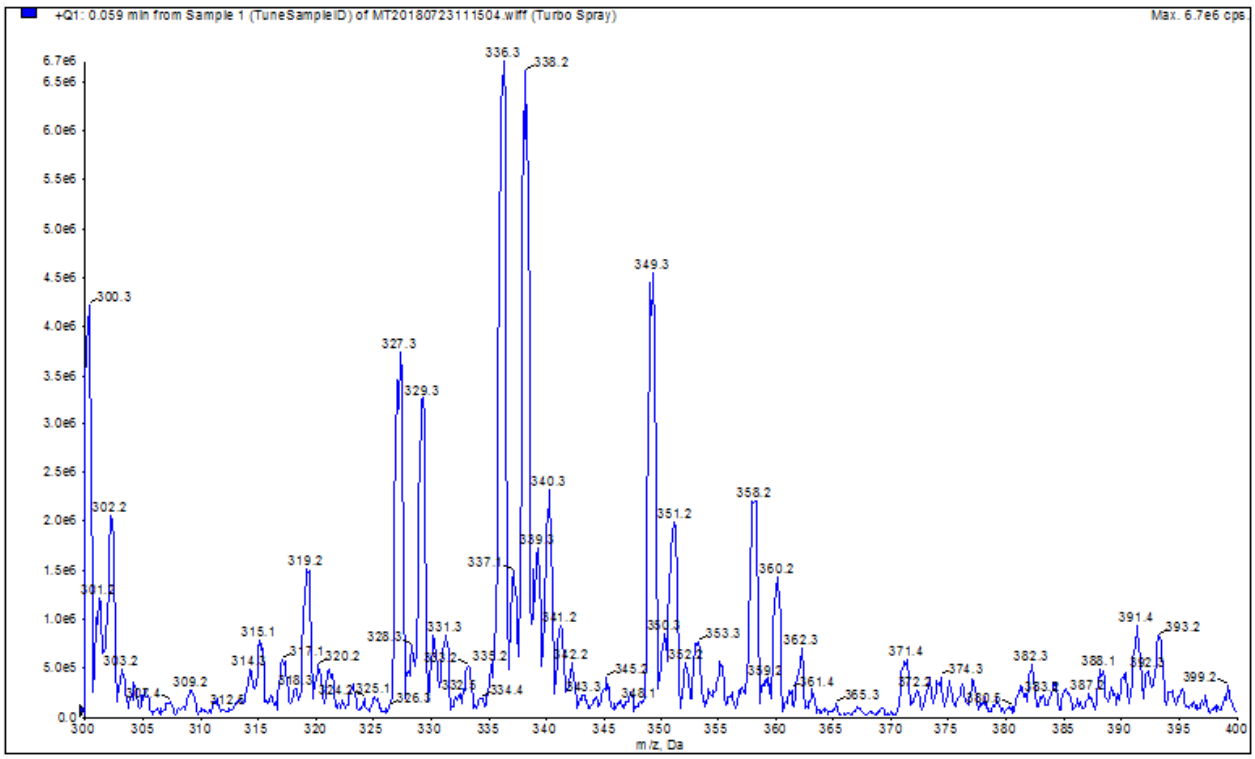


Figure A 61: Mass spectrum of zoxamide

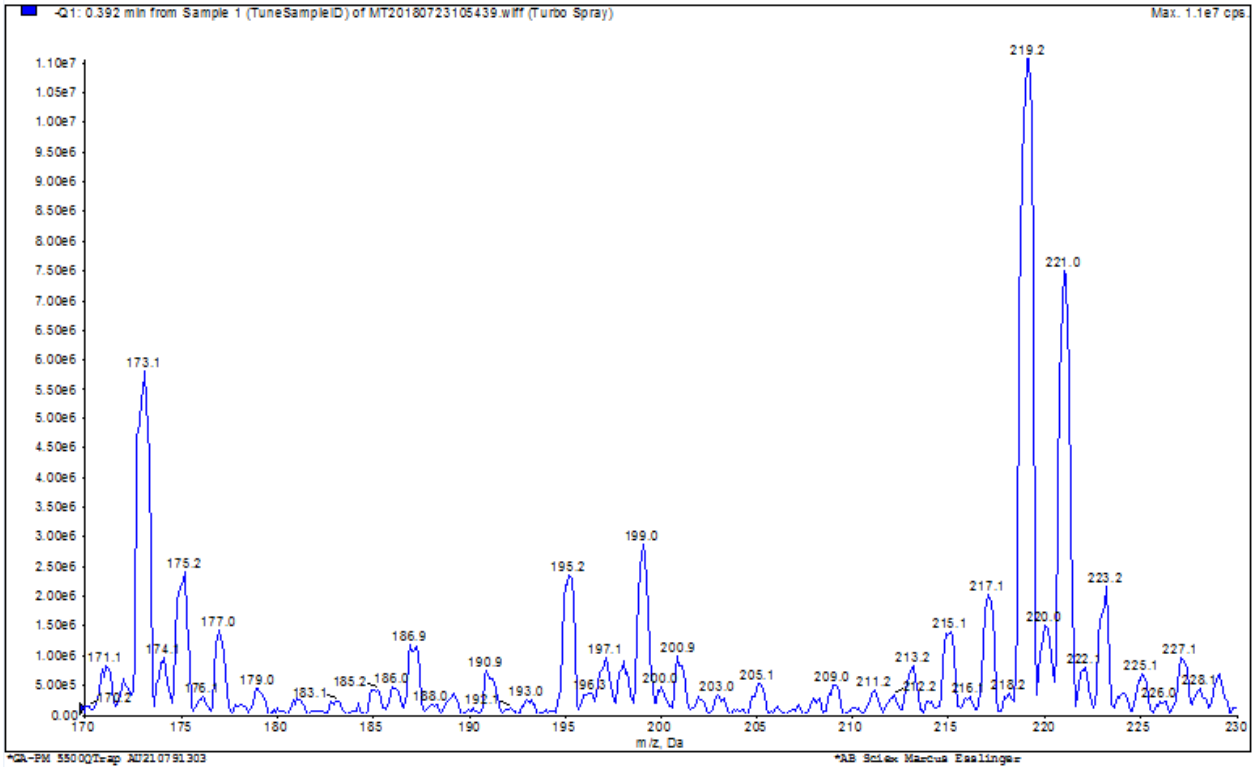


Figure A 62: Mass spectrum of RH-141452

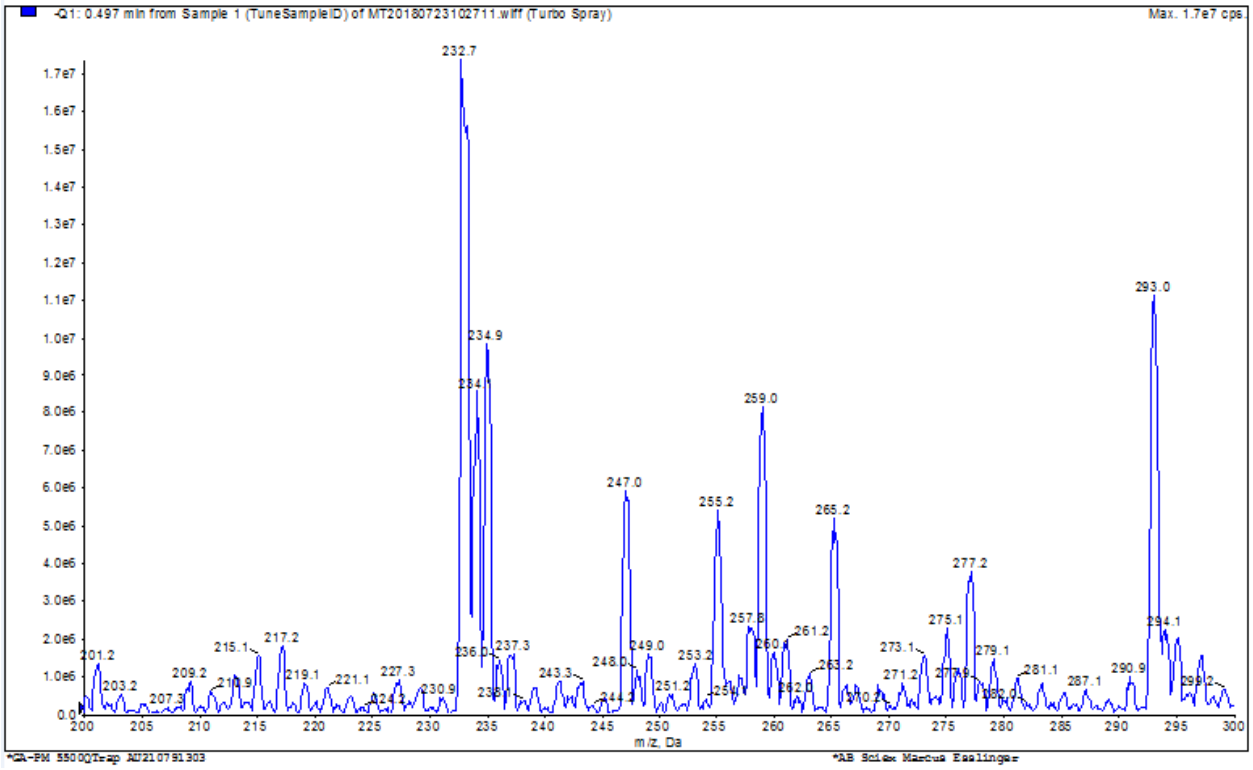


Figure A 63: Mass spectrum of RH-141455

Extraction efficiency

The extraction procedure used in this study was according to the QuEChERS method (validated for zoxamide in study No P 3114 G (RICHTER, S., PTRL Europe 2014)). This method was checked for its extraction efficiency in study AS362 (Hein, W., RLP AgroScience GmbH, Germany, 2014) in pea immature whole plant and dry peas. Therefore, the extraction efficiency was considered to be proven for dry and water containing plant material.

Conclusion

The applicability of a method (in analogy to the multiresidue method QuEChERS (EN 15662)) for analysis of residues of zoxamide isomers in different plant tissue matrices was tested, i.e. potato chips, potato flakes, pickled silverskin onions and potato tubers. The specimen extracts were analysed using liquid chromatography with mass selective detection (HPLC-MS/MS). The method was validated successfully according to SANCO/825/00 rev.8.1 (2010).

The method was proven to be specific, accurate and precise and good repeatability and recovery was found in all matrices. Therefore, this QuEChERS based method can be used for monitoring of zoxamide isomer residues in all tested matrix groups.

The applicability of the method for analysis of residues of zoxamide metabolites RH-141452 and RH-141455 was tested in the same matrices. The specimen extracts were also analysed using liquid chromatography with mass selective detection (HPLC-MS/MS). The method was validated successfully according to SANCO/825/00 rev.8.1 (2010) for metabolites RH-141452 and RH-141455, but deemed unsuitable for analysis of zoxamide itself.

The method was proven to be specific, accurate and precise and good repeatability and recovery was found in all matrices. Therefore, the method can be used for monitoring of zoxamide metabolites RH-141452 and RH-141455 residues in all tested matrix groups.

(Witte A. 2020)

A 2.1.2.1.2 Analytical method 2 - determination of zoxamide and its metabolites and their isomers

The method of Sala (2020) can be seen as primary enforcement/monitoring method for the determination of zoxamide (R/S and sum) and its metabolites (and isomers) in all matrices (high water content, acidic, high oil content and dry). Since it covers a range of analytes, it can serve as primary method for different combinations of zoxamide residues, as needed (see chapter 5.2.2 and 5.3.2).

A 2.1.2.1.2.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.2/02**
Report	Sala, A., 2020: Validation of an analytical method to determine zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities Gowan Crop Protection Ltd., UK LabAnalysis, Italy, Report No. BPL-STUDY-18-000085, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary

Principle of the method

The method developed allowes the determination of zoxamide and its mtetabolites via HPLC-MS/MS and HPLC-HRMS/MS in grape fruit, potato tuber, tomato fruit, cucumber fruit, and onion bulb raw agricultural and relevant processed commodities. It has been fully validated in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The extraction efficiency of the analytical method has been verified according to SANTE 2017/10632 rev. 3.

The homogenised specimen material (12.5 g each; 5 g in case of potato flakes) was extracted with a mixture of water/acetonitrile/methanol (20/40/40 v/v) acidified with 0.2% of HCOOH. The extraction was repeated twice on all samples except liquids (wine and grape juice); these matrices were analysed by direct injection after dilution with the extraction mixture. The 2 extracted fractions were pooled and combined and brought up to a final volume of 40 mL (20 mL for potato flakes) using the same solvent mixture. After centrifugation, the (purified) extracts were transferred to a glass vial for analyses using 2 HPLC/MS systems: a triple quadrupole HPLC-MS/MS and a high resolution HPLC-Orbitrap mass spectrometer. Typical HPLC conditions as well as typical Rf-values and MS spectra are summarised below.

It is possible to determine the analytes zoxamide (sum, R/S), RH-150721 (sum, R/S), RH-129151 (sum, R/S), RH-141288 (sum, R/S), RH-127450 (sum, R/S), RH-149736, RH-149737, RH-24549, RH-139432, RH-141452 and RH-141455.

All 5 pairs of chiral analytes were separated to receive their single enantiomers by chiral chromatography.

For metabolite RH-129151 - due to the lack of single isomer analytical standards - it was impossible to attribute the absolute configuration (R) and (S) to the measured peaks. Therefore, the 2 enantiomers were assigned in the following as "RH-129151 A" (the first enantiomer eluted in the chromatographic runs) and "RH-129151 B" (the second enantiomer eluted in the chromatographic runs).

The metabolites RH-141452, RH-141255 and RH-149737, which are known to form sugar conjugates, can be determined after an additional alkaline hydrolysis step according to the procedure of Steinborn et al. (2017). This step is based on QuEChERS method for the simultaneous determination of acidic pesticides, their esters and conjugates following alkaline hydrolysis. It allows to quantitatively hydrolyse potential conjugates of zoxamide metabolites. Following, the recoveries with and without this hydrolysis step can be compared.

Equipment Method A – Determination of analytes with a chiral centre (except RH-129151)

Instrument:	HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470
Column:	Agilent Poroshell 120 EC-C18 4µm 4.6 x 100 mm + Phenomenex Lux 5µm Amylose-2 250 x 4.6 mm
Mobile phase:	A: 2 mM of ammonium formate in LC-MS grade water B: 90% acetonitrile UPLC grade / 10% methanol Elution mixture composition (isocratic run): 40% mobile phase A, 60% mobile phase B
Flow rate:	1 mL/min
Column temp.:	40°C
Injection volume:	20 µL
Retention time:	(R)-zoxamide: approx.16.9 min (S)-zoxamide: approx. 15.4 min (R)-RH-141288: approx. 8.6 min (S)-RH-141288: approx. 8.0 min (R)-RH-150721: approx. 13.1 min (S)-RH-150721: approx. 16.1 min (R)-RH-127450: approx. 11.8 min (S)-RH-127450: approx. 12.8 min
Stop time:	22 min
Ionisation:	ESI (electrospray ionisation)
Ion mode:	

Analyte	Detection	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
(R)-Zoxamide	Primary	338	188.9	125	25
	Confirmatory	336	186.9		
(S)-Zoxamide	Primary	338	188.9	125	25
	Confirmatory	336	186.9		
(R)-RH-141288	Primary	320	206	125	13
	Confirmatory	318	204		
(S)-RH-141288	Primary	320	206	125	13
	Confirmatory	318	204		
(R)-RH-150721	Primary	320	188.9	125	21
	Confirmatory	318	186.9		
(S)-RH-150721	Primary	320	188.9	125	21
	Confirmatory	318	186.9		
(R)-RH-127450	Primary	302	159	135	45

	Confirmatory	302	187		25
(S)-RH-127450	Primary	302	159	135	45
	Confirmatory	302	187		25

Equipment Method B – Determination of RH-129151

This molecule is analysed both with triple quadrupole HPLC-MS/MS instrument and HPLC-HRMS orbitrap system, the 2 methods are reported hereunder:

HPLC-MS/MS (Triple quadrupole spectrometer)

Instrument: HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470
Column: Phenomenex Kinetex C18, 5µm, 2.1 x 50 mm + Waters Trefoil Amy1, 2.5 µm, 3 x 150 mm
Mobile phase: A: 0.1 % v/v formic acid in LC-MS grade water
B: 0.1% v/v formic acid in ethanol
Elution mixture composition (isocratic run): 24% mobile phase A, 76% mobile phase B
Flow rate: 0.35 mL/min
Column temp.: 40°C
Injection volume: 10 µL
Retention time: RH-129151 (A): approx. 7.4 min
RH-129151 (B): approx. 9.0 min
Stop time: 15 min
Ionisation: ESI (electrospray ionisation)

Ion mode:

Analyte	Detection	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
RH-129151 (A)	Primary	300	186	150	33
	Confirmatory	302	188		
RH-129151 (B)	Primary	300	186	150	33
	Confirmatory	302	188		

HPLC-HRMS (Orbitrap spectrometer)

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Phenomenex Kinetex C18, 5µm, 2.1 x 50 mm + Waters Trefoil Amy1, 2.5 µm, 3 x 150 mm
Mobile phase: A: 0.1 % v/v formic acid in LC-MS grade water
B: 0.1% v/v formic acid in ethanol
Elution mixture composition (isocratic run): 24% mobile phase A , 76% mobile phase B
Flow rate: 0.35 mL/min
Column temp.: 40°C
Injection volume: 8 µL
Retention time: RH-129151 (A): approx. 6.8 min
RH-129151 (B): approx. 8.2 min
Stop time: 15 min
Ionisation: Parallel Reaction Monitoring (PRM), positive

Ion mode:

Analyte	Detection	Precursor ion	Product ion	Collision energy (V)
RH-129151 (A)	Primary	300.0553	185.9872	40
	Confirmatory	302.0523	187.9842	
RH-129151 (B)	Primary	300.0553	185.9872	40
	Confirmatory	302.0523	187.9842	

Equipment Method C – Determination of non-chiral analytes (except onion and potato tuber and processed commodities)

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Waters Xselect HSS PFP, 3.5 µm, 4.6 x 150 mm
Mobile phase: Solvent A: 0.2% v/v formic acid in LC-MS grade water
Solvent B: 0.1% v/v formic acid in methanol

Time (minutes)	Solvent A%	Solvent B%
0	80	20
2	80	20
10	0	100
13	0	100
13.1	80	20
16	80	20

Flow rate: 0.6 mL/min
Column temp.: 30°C
Injection volume: 4 µL
Retention time: RH-141452: approx. 10.28 min
RH-24549: approx. 12.05 min
RH-139432: approx. 11.37 min
RH-149736: approx. 9.09 min
RH-149737: approx. 6.64 min
Stop time: 16 min
Ionisation: ESI (electrospray ionisation)

Ion mode:

Analyte	Detection	Parent and Fragment ions (m/z)	Fragment ions (m/Z)	Collision energy (V)	Polarity
RH-141452	Primary	218.9621 (SIM)		/	
	Confirmatory	218.9621	144.9617	20	
RH-24549	Primary	202.9672 (SIM)		/	
	Confirmatory	202.9672	158.9774	20	
RH-139432	Primary	203.9977 (SIM)		/	Positive
	Confirmatory	203.9977	186.9709	45	
RH-149736	Primary	219.9927 (SIM)		/	
	Confirmatory	219.9927	158.9761	45	
RH-149737	Primary	187.9675		/	Negative
	Confirmatory	189.9446		/	

The analyses of the hydrolysed samples for the determination of the total (free + conjugated) RH-141452 and RH-149737 were carried out with this analytical method (except potato - tuber and processed commodities - samples).

Equipment Method D - Determination of non-chiral analytes in onion and potato tuber and processed commodities

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Waters Xselect HSS PFP, 3.5 µm, 4.6 x 150 mm
Mobile phase: Solvent A: 0.2% v/v formic acid in LC-MS grade water
Solvent B: 0.1% v/v formic acid in methanol

Time (minutes)	Solvent A%	Solvent B%
0	80	20
2	80	20
10	0	100
13	0	100
13.1	80	20
16	80	20

Flow rate: 0.6 mL/min
Column temp.: 30°C
Injection volume: 4 µL
Retention time: RH-141455: approx. 9.29 min
RH-141452: approx. 10.28 min
RH-24549: approx. 12.05 min
Stop time: 16 min
Ionisation: ESI (electrospray ionisation), negative

Ion mode:

Analyte	Detection	Parent and fragment ions (m/z)	Fragment ions (m/Z)	Collision energy (V)	Polarity
RH-141455	Primary	232.9414 (SIM)		/	Negative
	Confirmatory	232.9414	188.9516	20	
RH-141452	Primary	218.9621 (SIM)		/	
	Confirmatory	218.9621	144.9617	20	
RH-24549	Primary	202.9672 (SIM)		/	
	Confirmatory	202.9672	158.9774	20	

The analyses of the hydrolysed samples for the determination of the total (free + conjugated) RH-141452 and RH-141455 in onion and potato tuber and processed commodities were carried out with this analytical method.

Results and discussion

Table A 147: Recovery results in grape fruits

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamide: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	100.1	2.4	**100.6**	**1.79**
	0.1 (10x LOQ)	101.1	1.0		
(S)-Zoxamide	0.01 (LOQ)	99.2	0.9	**99.1**	**0.87**
	0.1 (10x LOQ)	98.9	1.0		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamide sum	0.01 (LOQ)	99.6	1.1	**99.8**	**0.78**
	0.1 (10x LOQ)	100.0	0.2		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	93.4	4.6	**95.8**	**4.22**
	0.1 (10x LOQ)	98.2	2.1		
(S)-Zoxamide	0.01 (LOQ)	94.3	1.3	**96.4**	**2.89**
	0.1 (10x LOQ)	98.4	2.4		
Zoxamide sum	0.01 (LOQ)	93.9	2.8	**96.1**	**3.39**
	0.1 (10x LOQ)	98.3	2.2		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	98.1	1.9	**99.3**	**2.26**
	0.1 (10x LOQ)	100.6	2.0		
(S)-RH-150721	0.01 (LOQ)	104.3	1.1	**104.8**	**1.24**
	0.1 (10x LOQ)	105.3	1.3		
RH-150721 sum	0.01 (LOQ)	101.1	1.1	**102.0**	**1.53**
	0.1 (10x LOQ)	102.9	1.5		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	100.0	2.8	**102.3**	**3.12**
	0.1 (10x LOQ)	104.6	1.4		
(S)-RH-150721	0.01 (LOQ)	101.7	1.5	**103.4**	**2.17**
	0.1 (10x LOQ)	105.1	1.3		
RH-150721 sum	0.01 (LOQ)	100.8	1.7	**102.8**	**2.45**
	0.1 (10x LOQ)	104.8	1.1		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	91.6	2.1	**88.3**	**4.6**
	0.1 (10x LOQ)	85.0	3.1		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	87.3	4.4	**86.4**	**4.4**
	0.1 (10x LOQ)	85.5	4.6		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	91.4	2.2	**88.7**	**4.4**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	86.1	4.3		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	105.5	3.3	**93.9**	**13.4**
	0.1 (10x LOQ)	82.4	3.7		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	93.0	1.6	**89.2**	**5.1**
	0.1 (10x LOQ)	85.4	3.3		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	95.0	2.0	**88.6**	**8.1**
	0.1 (10x LOQ)	82.3	4.2		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	79.1	6.4	**79.0**	**5.44**
	0.1 (10x LOQ)	78.9	5.0		
RH-129151 (B)	0.01 (LOQ)	79.9	6.2	**77.8**	**5.52**
	0.1 (10x LOQ)	75.7	3.2		
RH-129151 (sum)	0.01 (LOQ)	77.8	6.4	**78.7**	**5.68**
	0.1 (10x LOQ)	79.6	5.3		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	76.5	6.5	**78.4**	**6.53**
	0.1 (10x LOQ)	80.3	6.2		
RH-129151 (B)	0.01 (LOQ)	76.5	9.6	**76.6**	**8.11**
	0.1 (10x LOQ)	76.7	7.5		
RH-159151 (sum)	0.01 (LOQ)	78.2	7.7	**77.2**	**6.38**
	0.1 (10x LOQ)	76.2	5.2		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	89.0	2.4	**90.6**	**2.65**
	0.1 (10x LOQ)	92.3	1.5		
(S)-RH-141288	0.01 (LOQ)	98.7	2.6	**99.6**	**2.05**
	0.1 (10x LOQ)	100.4	1.1		
RH-141288 (sum)	0.01 (LOQ)	93.7	2.4	**95.0**	**2.19**
	0.1 (10x LOQ)	96.2	1.0		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	86.2	2.0	**89.6**	**4.24**
	0.1 (10x LOQ)	93.0	1.2		
(S)-RH-141288	0.01 (LOQ)	98.3	3.7	**99.9**	**3.07**
	0.1 (10x LOQ)	101.4	1.3		
RH-141288 (sum)	0.01 (LOQ)	92.1	1.8	**94.6**	**3.08**
	0.1 (10x LOQ)	97.1	1.0		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	97.5	3.3	**100.2**	**3.73**
	0.1 (10x LOQ)	102.9	1.7		
(S)-RH-127450	0.01 (LOQ)	99.7	1.7	**102.5**	**3.36**
	0.1 (10x LOQ)	105.3	2.0		
RH-127450 (sum)	0.01 (LOQ)	98.6	2.2	**101.3**	**3.43**
	0.1 (10x LOQ)	104.1	1.8		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	100.3	0.7	**101.2**	**1.46**
	0.1 (10x LOQ)	102.0	1.5		
(S)-RH-127450	0.01 (LOQ)	100.4	1.7	**101.5**	**1.89**
	0.1 (10x LOQ)	102.7	1.4		
RH-127450 (sum)	0.01 (LOQ)	100.3	0.8	**101.3**	**1.51**
	0.1 (10x LOQ)	102.4	1.4		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	104.5	3.3	**94.4**	**11.7**
	0.1 (10x LOQ)	84.2	3.2		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	100.3	4.0	**92.2**	**10.1**
	0.1 (10x LOQ)	84.1	4.4		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	78.0	4.6	**80.2**	**4.9**
	0.1 (10x LOQ)	82.4	3.8		
RH-149737: qualifier transition 189.9647 (SIM)					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-149737	0.01 (LOQ)	80.6	3.2	**81.0**	**4.3**
	0.1 (10x LOQ)	81.3	1.4		

Table A 148: Recovery results in juice

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	106.4	1.3	**105.8**	**2.24**
	0.1 (10x LOQ)	105.1	3.0		
(S)-Zoxamide	0.01 (LOQ)	106.9	2.3	**106.7**	**1.87**
	0.1 (10x LOQ)	106.4	1.6		
Zoxamide sum	0.01 (LOQ)	106.6	0.8	**106.2**	**1.20**
	0.1 (10x LOQ)	105.8	1.5		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	107.3	2.1	**106.4**	**2.03**
	0.1 (10x LOQ)	105.5	1.7		
(S)-Zoxamide	0.01 (LOQ)	104.9	2.0	**106.4**	**2.11**
	0.1 (10x LOQ)	107.8	1.4		
Zoxamide sum	0.01 (LOQ)	106.1	0.9	**106.4**	**0.95**
	0.1 (10x LOQ)	106.6	1.0		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	100.8	2.9	**103.2**	**3.31**
	0.1 (10x LOQ)	105.5	1.8		
(S)-RH-150721	0.01 (LOQ)	101.9	2.0	**103.9**	**2.72**
	0.1 (10x LOQ)	105.9	1.7		
RH-150721 sum	0.01 (LOQ)	101.3	2.1	**103.5**	**2.81**
	0.1 (10x LOQ)	105.7	1.5		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	100.1	4.6	**103.7**	**4.80**
	0.1 (10x LOQ)	107.3	1.5		
(S)-RH-150721	0.01 (LOQ)	107.4	1.4	**104.5**	**3.65**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	101.6	3.1		
RH-150721 sum	0.01 (LOQ)	103.7	2.7	**104.1**	**2.26**
	0.1 (10x LOQ)	104.5	1.9		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	98.8	0.9	**97.2**	**2.7**
	0.1 (10x LOQ)	95.7	3.0		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	94.8	4.0	**95.2**	**2.8**
	0.1 (10x LOQ)	95.6	1.2		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	105.0	1.3	**102.4**	**2.9**
	0.1 (10x LOQ)	99.7	0.7		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	101.3	1.5	**98.8**	**4.1**
	0.1 (10x LOQ)	96.4	4.7		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	105.3	2.4	**102.1**	**3.8**
	0.1 (10x LOQ)	98.9	1.4		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	104.9	2.5	**102.2**	**3.4**
	0.1 (10x LOQ)	99.4	1.4		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	93.4	11.4	**98.4**	**10.35**
	0.1 (10x LOQ)	103.4	7.2		
RH-129151 (B)	0.01 (LOQ)	88.8	13.0	**96.9**	**13.02**
	0.1 (10x LOQ)	105.1	7.3		
RH-129151 (sum)	0.01 (LOQ)	93.9	8.8	**96.9**	**8.20**
	0.1 (10x LOQ)	99.9	7.2		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	94.4	6.4	**95.4**	**6.71**
	0.1 (10x LOQ)	96.3	7.6		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-129151 (B)	0.01 (LOQ)	92.0	8.1	**95.4**	**8.08**
	0.1 (10x LOQ)	98.7	7.1		
RH-159151 (sum)	0.01 (LOQ)	90.4	10.4	**96.1**	**10.35**
	0.1 (10x LOQ)	101.9	7.0		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	102.2	2.5	**101.7**	**1.85**
	0.1 (10x LOQ)	101.2	1.0		
(S)-RH-141288	0.01 (LOQ)	100.0	1.5	**101.5**	**2.50**
	0.1 (10x LOQ)	102.9	2.6		
RH-141288 (sum)	0.01 (LOQ)	101.1	1.9	**101.6**	**1.52**
	0.1 (10x LOQ)	102.0	1.1		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	101.9	1.4	**101.6**	**1.36**
	0.1 (10x LOQ)	101.4	1.4		
(S)-RH-141288	0.01 (LOQ)	101.2	3.3	**103.5**	**3.14**
	0.1 (10x LOQ)	105.7	0.4		
RH-141288 (sum)	0.01 (LOQ)	101.6	2.0	**102.5**	**1.71**
	0.1 (10x LOQ)	103.5	0.7		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	109.0	0.7	**108.8**	**0.97**
	0.1 (10x LOQ)	108.7	1.3		
(S)-RH-127450	0.01 (LOQ)	109.3	1.4	**108.7**	**1.49**
	0.1 (10x LOQ)	108.2	1.6		
RH-127450 (sum)	0.01 (LOQ)	109.1	0.9	**108.8**	**1.03**
	0.1 (10x LOQ)	108.4	1.2		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	105.7	1.6	**106.6**	**1.53**
	0.1 (10x LOQ)	107.6	0.8		
(S)-RH-127450	0.01 (LOQ)	104.5	1.1	**105.6**	**2.08**
	0.1 (10x LOQ)	106.8	2.3		
RH-127450 (sum)	0.01 (LOQ)	105.1	0.7	**106.1**	**1.38**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	107.2	1.2		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	96.8	1.9	**96.6**	**1.7**
	0.1 (10x LOQ)	96.4	1.7		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	99.6	3.4	**98.0**	**3.1**
	0.1 (10x LOQ)	96.4	1.7		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	104.6	3.2	**102.2**	**3.4**
	0.1 (10x LOQ)	99.8	0.9		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	103.0	2.6	**101.6**	**2.7**
	0.1 (10x LOQ)	100.1	2.0		

Table A 149: Recovery results in wine

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	106.2	3.3	**105.9**	**2.58**
	0.1 (10x LOQ)	105.7	1.9		
(S)-Zoxamide	0.01 (LOQ)	101.4	5.1	**103.0**	**3.87**
	0.1 (10x LOQ)	104.7	1.6		
Zoxamide sum	0.01 (LOQ)	103.8	4.1	**104.5**	**3.05**
	0.1 (10x LOQ)	105.2	1.7		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	94.9	2.3	**100.0**	**5.79**
	0.1 (10x LOQ)	105.1	2.2		
(S)-Zoxamide	0.01 (LOQ)	98.5	1.3	**101.2**	**3.08**
	0.1 (10x LOQ)	104.0	1.2		
Zoxamide sum	0.01 (LOQ)	96.7	1.6	**100.6**	**4.40**
	0.1 (10x LOQ)	104.5	1.7		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	86.3	4.9	**96.6**	**9.06**
	0.1 (10x LOQ)	101.0	2.9		
(S)-RH-150721	0.01 (LOQ)	104.1	2.9	**105.7**	**2.88**
	0.1 (10x LOQ)	107.2	2.2		
RH-150721 sum	0.01 (LOQ)	95.1	3.3	**99.6**	**5.44**
	0.1 (10x LOQ)	104.1	2.4		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	90.0	3.0	**96.1**	**7.19**
	0.1 (10x LOQ)	102.2	2.6		
(S)-RH-150721	0.01 (LOQ)	99.8	2.2	**103.6**	**4.48**
	0.1 (10x LOQ)	107.5	2.4		
RH-150721 sum	0.01 (LOQ)	94.8	1.8	**99.8**	**5.59**
	0.1 (10x LOQ)	104.8	2.1		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	99.5	2.2	**98.8**	**3.2**
	0.1 (10x LOQ)	98.1	4.1		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	100.2	3.1	**100.1**	**3.0**
	0.1 (10x LOQ)	100.0	3.3		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	101.4	2.3	**100.4**	**2.8**
	0.1 (10x LOQ)	99.5	3.2		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	108.4	1.1	**102.2**	**6.7**
	0.1 (10x LOQ)	96.0	2.8		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	103.4	2.0	**99.7**	**4.5**
	0.1 (10x LOQ)	96.0	2.9		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	102.1	5.5	**100.3**	**4.7**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	98.5	3.2		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	96.8	3.2	**88.0**	**11.26**
	0.1 (10x LOQ)	79.2	5.3		
RH-129151 (B)	0.01 (LOQ)	96.7	3.9	**87.8**	**11.45**
	0.1 (10x LOQ)	78.8	4.6		
RH-129151 (sum)	0.01 (LOQ)	97.6	2.7	**87.8**	**12.29**
	0.1 (10x LOQ)	78.0	5.2		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	98.4	2.4	**87.7**	**13.43**
	0.1 (10x LOQ)	76.9	5.4		
RH-129151 (B)	0.01 (LOQ)	98.2	2.0	**88.0**	**12.55**
	0.1 (10x LOQ)	77.9	4.5		
RH-159151 (sum)	0.01 (LOQ)	97.5	2.8	**87.9**	**11.95**
	0.1 (10x LOQ)	78.3	4.5		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	98.6	0.8	**99.3**	**1.13**
	0.1 (10x LOQ)	100.0	1.1		
(S)-RH-141288	0.01 (LOQ)	97.5	5.9	**100.7**	**5.04**
	0.1 (10x LOQ)	103.8	0.8		
RH-141288 (sum)	0.01 (LOQ)	98.1	2.7	**100.0**	**2.68**
	0.1 (10x LOQ)	101.8	0.7		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	97.8	2.5	**98.9**	**2.34**
	0.1 (10x LOQ)	100.0	1.7		
(S)-RH-141288	0.01 (LOQ)	95.1	3.5	**98.8**	**5.15**
	0.1 (10x LOQ)	102.4	3.7		
RH-141288 (sum)	0.01 (LOQ)	96.5	2.6	**98.8**	**3.34**
	0.1 (10x LOQ)	101.2	2.0		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	101.1	3.2	**104.0**	**3.91**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	107.0	2.1		
(S)-RH-127450	0.01 (LOQ)	102.9	1.0	**104.8**	**2.29**
	0.1 (10x LOQ)	106.8	1.4		
RH-127450 (sum)	0.01 (LOQ)	102.0	1.5	**104.4**	**2.91**
	0.1 (10x LOQ)	106.9	1.7		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	103.1	2.0	**105.4**	**2.86**
	0.1 (10x LOQ)	107.7	1.7		
(S)-RH-127450	0.01 (LOQ)	104.7	3.1	**105.4**	**2.64**
	0.1 (10x LOQ)	106.1	2.2		
RH-127450 (sum)	0.01 (LOQ)	103.9	2.4	**105.4**	**2.43**
	0.1 (10x LOQ)	106.9	1.6		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	96.9	1.4	**96.7**	**3.5**
	0.1 (10xLOQ)	96.6	5.0		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	97.4	6.4	**97.6**	**5.1**
	0.1 (10x LOQ)	97.9	4.3		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	105.6	3.1	**103.5**	**3.7**
	0.1 (10x LOQ)	101.3	3.3		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	102.1	6.3	**102.3**	**4.3**
	0.1 (10x LOQ)	102.5	1.4		

Table A 150: Recovery results in raisins

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	91.4	4.8	**93.8**	**5.00**
	0.1 (10x LOQ)	96.2	4.2		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
(S)-Zoxamide	0.01 (LOQ)	90.4	5.4	**94.4**	**6.39**
	0.1 (10x LOQ)	98.5	4.2		
Zoxamide sum	0.01 (LOQ)	90.9	4.3	**94.1**	**5.38**
	0.1 (10x LOQ)	97.3	4.1		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	89.6	5.4	**93.5**	**6.39**
	0.1 (10x LOQ)	97.4	4.4		
(S)-Zoxamide	0.01 (LOQ)	93.9	3.8	**95.8**	**4.31**
	0.1 (10x LOQ)	97.7	4.2		
Zoxamide sum	0.01 (LOQ)	91.7	4.2	**94.6**	**5.14**
	0.1 (10x LOQ)	97.5	4.3		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	92.1	4.7	**95.1**	**4.91**
	0.1 (10x LOQ)	98.1	3.0		
(S)-RH-150721	0.01 (LOQ)	97.9	1.7	**101.1**	**4.28**
	0.1 (10x LOQ)	104.3	3.5		
RH-150721 sum	0.01 (LOQ)	95.0	3.1	**98.1**	**4.45**
	0.1 (10x LOQ)	101.2	3.2		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	93.2	3.8	**95.5**	**4.13**
	0.1 (10x LOQ)	97.9	2.9		
(S)-RH-150721	0.01 (LOQ)	107.6	2.0	**104.9**	**3.41**
	0.1 (10x LOQ)	102.2	2.4		
RH-150721 sum	0.01 (LOQ)	100.3	2.7	**100.2**	**2.35**
	0.1 (10x LOQ)	100.0	2.2		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	85.6	4.1	**91.6**	**7.62**
	0.1 (10x LOQ)	97.6	2.8		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	84.8	3.1	**92.3**	**9.21**
	0.1 (10x LOQ)	99.8	3.9		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	83.1	6.9	**88.4**	**8.32**
	0.1 (10x LOQ)	93.8	4.5		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	92.0	7.2	**89.7**	**6.52**
	0.1 (10x LOQ)	87.3	5.0		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	76.9	7.9	**85.8**	**12.19**
	0.1 (10x LOQ)	94.6	3.9		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	90.4	5.5	**90.4**	**4.25**
	0.1 (10x LOQ)	90.4	3.1		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	76.9	9.3	**81.8**	**8.79**
	0.1 (10x LOQ)	84.0	8.4		
RH-129151 (B)	0.01 (LOQ)	76.5	10.6	**80.6**	**10.45**
	0.1 (10x LOQ)	84.7	8.5		
RH-129151 (sum)	0.01 (LOQ)	81.4	8.9	**83.2**	**8.47**
	0.1 (10x LOQ)	85.0	8.4		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	83.1	8.9	**84.6**	**8.55**
	0.1 (10x LOQ)	86.0	8.8		
RH-129151 (B)	0.01 (LOQ)	82.2	11.4	**83.6**	**8.83**
	0.1 (10x LOQ)	85.0	6.4		
RH-159151 (sum)	0.01 (LOQ)	79.4	10.8	**82.1**	**9.12**
	0.1 (10x LOQ)	84.8	6.8		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	97.7	2.4	**97.4**	**3.03**
	0.1 (10x LOQ)	97.1	3.8		
(S)-RH-141288	0.01 (LOQ)	95.1	7.1	**95.7**	**6.10**
	0.1 (10x LOQ)	96.3	5.7		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141288 (sum)	0.01 (LOQ)	95.5	3.9	**96.6**	**3.44**
	0.1 (10x LOQ)	96.7	3.3		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	82.0	4.1	**90.4**	**10.24**
	0.1 (10x LOQ)	98.8	2.2		
(S)-RH-141288	0.01 (LOQ)	93.1	3.2	**89.6**	**9.75**
	0.1 (10x LOQ)	96.1	8.0		
RH-141288 (sum)	0.01 (LOQ)	82.5	2.6	**90.0**	**9.39**
	0.1 (10x LOQ)	97.5	4.2		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	86.4	5.2	**92.6**	**8.52**
	0.1 (10x LOQ)	98.8	4.9		
(S)-RH-127450	0.01 (LOQ)	86.0	5.5	**93.2**	**9.46**
	0.1 (10x LOQ)	100.5	4.6		
RH-127450 (sum)	0.01 (LOQ)	86.2	5.3	**92.9**	**8.97**
	0.1 (10x LOQ)	99.7	4.7		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	87.0	5.9	**92.4**	**7.87**
	0.1 (10x LOQ)	97.7	4.7		
(S)-RH-127450	0.01 (LOQ)	85.8	6.3	**93.0**	**9.62**
	0.1 (10x LOQ)	100.2	4.7		
RH-127450 (sum)	0.01 (LOQ)	86.4	6.0	**92.7**	**8.68**
	0.1 (10x LOQ)	98.9	4.6		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	91.9	3.3	**94.4**	**4.47**
	0.1 (10x LOQ)	96.9	4.0		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	95.1	6.6	**97.3**	**5.58**
	0.1 (10x LOQ)	99.4	3.9		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	74.7	6.6	**82.8**	**11.41**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	91.0	3.7		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	76.5	9.4	**83.5**	**11.19**
	0.1 (10x LOQ)	90.4	5.4		

Table A 151: Recovery results in potato tubers

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	102.0	1.28	**104.9**	**3.5**
	0.1 (10x LOQ)	107.8	0.9		
(S)-Zoxamide	0.01 (LOQ)	108.2	0.9	**107.2**	**2.0**
	0.1 (10x LOQ)	106.3	2.5		
Zoxamide sum	0.01 (LOQ)	105.1	1.7	**106.1**	**1.83**
	0.1 (10x LOQ)	107.0	1.7		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	102.3	3.3	**105.8**	**4.1**
	0.1 (10x LOQ)	19.3	0.8		
(S)-Zoxamide	0.01 (LOQ)	107.1	2.3	**107.7**	**1.7**
	0.1 (10x LOQ)	108.3	0.5		
Zoxamide sum	0.01 (LOQ)	104.8	2.4	**106.8**	**2.55**
	0.1 (10x LOQ)	108.8	0.6		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	87.4	6.2	**85.3**	**5.6**
	0.1 (10x LOQ)	83.2	4.1		
(S)-RH-150721	0.01 (LOQ)	92.1	4.2	**92.1**	**4.7**
	0.1 (10x LOQ)	92.2	5.7		
RH-150721 sum	0.01 (LOQ)	89.8	4.8	**88.7**	**4.49**
	0.1 (10x LOQ)	87.6	4.3		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	87.1	5.3	**84.7**	**5.2**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	82.3	3.6		
(S)-RH-150721	0.01 (LOQ)	97.8	5.3	**94.7**	**6.1**
	0.1 (10x LOQ)	91.7	5.4		
RH-150721 sum	0.01 (LOQ)	92.5	5.1	**89.7**	**5.51**
	0.1 (10x LOQ)	86.9	4.2		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	91.2	3.1	**94.5**	**4.5**
	0.1 (10x LOQ)	97.8	2.2		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	88.1	0.6	**92.7**	**5.3**
	0.1 (10x LOQ)	97.2	1.7		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	84.3	1.7	**87.6**	**4.2**
	0.1 (10x LOQ)	90.8	1.7		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	89.5	2.7	**89.2**	**2.6**
	0.1 (10x LOQ)	88.9	2.9		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	72.7	3.1	**86.6**	**17.0**
	0.1 (10x LOQ)	100.5	1.3		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	74.7	5.7	**85.9**	**14.4**
	0.1 (10x LOQ)	97.2	3.0		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	100.9	3.1	**103.2**	**3.81**
	0.1 (10x LOQ)	105.5	3.2		
RH-129151 (B)	0.01 (LOQ)	102.1	1.5	**102.9**	**2.94**
	0.1 (10x LOQ)	103.6	4.0		
RH-129151 (sum)	0.01 (LOQ)	101.5	2.2	**103.0**	**2.20**
	0.1 (10x LOQ)	104.6	0.7		
RH-129151: qualifier transition 300.0523 → 185.9842					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-129151 (A)	0.01 (LOQ)	106.1	1.9	**105.5**	**1.66**
	0.1 (10x LOQ)	104.8	1.4		
RH-129151 (B)	0.01 (LOQ)	106.2	1.1	**106.0**	**1.16**
	0.1 (10x LOQ)	105.9	1.3		
RH-159151 (sum)	0.01 (LOQ)	106.0	1.2	**105.7**	**1.13**
	0.1 (10x LOQ)	105.4	1.1		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	98.7	3.3	**100.4**	**3.5**
	0.1 (10x LOQ)	102.1	3.0		
(S)-RH-141288	0.01 (LOQ)	104.9	3.4	**105.1**	**2.3**
	0.1 (10x LOQ)	105.3	0.7		
RH-141288 (sum)	0.01 (LOQ)	101.6	2.8	**102.7**	**2.35**
	0.1 (10x LOQ)	103.7	1.5		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	103.0	2.1	**102.6**	**1.7**
	0.1 (10x LOQ)	102.3	1.2		
(S)-RH-141288	0.01 (LOQ)	106.1	3.6	**106.6**	**2.6**
	0.1 (10x LOQ)	107.2	1.2		
RH-141288 (sum)	0.01 (LOQ)	104.6	1.8	**104.6**	**1.46**
	0.1 (10x LOQ)	104.7	1.2		

Table A 152: Recovery results in potato flakes

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	91.2	3.0	**89.7**	**4.45**
	0.1 (10x LOQ)	88.2	5.5		
(S)-Zoxamide	0.01 (LOQ)	91.8	2.6	**90.7**	**4.53**
	0.1 (10x LOQ)	89.7	6.1		
Zoxamide sum	0.01 (LOQ)	91.5	1.6	**90.2**	**4.20**
	0.1 (10x LOQ)	89.0	5.8		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	93.2	3.9	**90.1**	**5.86**
	0.1 (10x LOQ)	87.0	5.8		
(S)-Zoxamide	0.01 (LOQ)	90.8	3.5	**89.9**	**4.71**
	0.1 (10x LOQ)	88.9	6.0		
Zoxamide sum	0.01 (LOQ)	92.0	3.5	**90.0**	**5.04**
	0.1 (10x LOQ)	88.0	5.8		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	94.8	1.0	**89.3**	**7.39**
	0.1 (10x LOQ)	83.7	5.4		
(S)-RH-150721	0.01 (LOQ)	94.7	1.8	**89.9**	**6.76**
	0.1 (10x LOQ)	85.0	5.3		
RH-150721 sum	0.01 (LOQ)	94.8	1.1	**89.6**	**7.04**
	0.1 (10x LOQ)	84.4	5.3		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	95.3	1.0	**89.6**	**7.55**
	0.1 (10x LOQ)	84.0	5.6		
(S)-RH-150721	0.01 (LOQ)	103.8	1.2	**98.5**	**6.55**
	0.1 (10x LOQ)	93.3	5.1		
RH-150721 sum	0.01 (LOQ)	99.5	0.5	**94.0**	**6.99**
	0.1 (10x LOQ)	88.6	5.4		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	84.8	1.3	**81.9**	**5.20**
	0.1 (10x LOQ)	79.0	5.4		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.4	0.8	**84.9**	**11.11**
	0.1 (10x LOQ)	76.5	5.9		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	86.3	0.8	**82.2**	**6.34**
	0.1 (10x LOQ)	78.1	5.6		
RH-141452: qualifier transition 218.9621 → 144.9617					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452	0.01 (LOQ)	93.9	0.8	**85.2**	**11.25**
	0.1 (10xLOQ)	76.6	5.4		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	82.3	1.1	**77.2**	**8.41**
	0.1 (10x LOQ)	72.0	7.4		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	101.0	4.0	**86.2**	**18.77**
	0.1 (10x LOQ)	71.4	7.0		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	86.5	2.6	**86.4**	**2.13**
	0.1 (10x LOQ)	86.4	1.8		
RH-129151 (B)	0.01 (LOQ)	74.8	4.8	**81.0**	**8.79**
	0.1 (10x LOQ)	87.2	2.5		
RH-129151 (sum)	0.01 (LOQ)	80.8	3.3	**83.8**	**4.47**
	0.1 (10x LOQ)	86.8	1.5		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	77.9	3.6	**81.8**	**5.82**
	0.1 (10x LOQ)	85.6	0.8		
RH-129151 (B)	0.01 (LOQ)	73.3	3.9	**80.5**	**9.99**
	0.1 (10x LOQ)	87.7	3.1		
RH-159151 (sum)	0.01 (LOQ)	75.6	4.4	**81.1**	**7.76**
	0.1 (10x LOQ)	86.7	1.4		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	93.7	3.9	**90.1**	**6.53**
	0.1 (10x LOQ)	86.4	6.4		
(S)-RH-141288	0.01 (LOQ)	785	2.6	**75.3**	**5.73**
	0.1 (10x LOQ)	72.0	4.6		
RH-141288 (sum)	0.01 (LOQ)	86.3	2.6	**82.8**	**5.83**
	0.1 (10x LOQ)	79.4	5.3		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	85.2	4.2	**85.9**	**4.62**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	86.6	5.4		
(S)-RH-141288	0.01 (LOQ)	74.7	2.7	**73.2**	**3.79**
	0.1 (10x LOQ)	71.7	3.9		
RH-141288 (sum)	0.01 (LOQ)	80.1	1.8	**79.7**	**3.12**
	0.1 (10x LOQ)	79.4	4.3		

Table A 153: Recovery results in fried potatoes

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	80.3	2.5	**77.2**	**4.68**
	0.1 (10x LOQ)	74.1	1.8		
(S)-Zoxamide	0.01 (LOQ)	79.8	4.5	**77.3**	**4.76**
	0.1 (10x LOQ)	74.7	1.5		
Zoxamide sum	0.01 (LOQ)	80.0	3.5	**77.2**	**4.64**
	0.1 (10x LOQ)	74.4	1.6		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	78.4	2.6	**76.3**	**3.56**
	0.1 (10x LOQ)	74.2	1.6		
(S)-Zoxamide	0.01 (LOQ)	77.4	4.6	**76.6**	**3.48**
	0.1 (10x LOQ)	75.7	1.5		
Zoxamide sum	0.01 (LOQ)	77.9	3.5	**76.4**	**3.29**
	0.1 (10x LOQ)	74.9	1.4		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	84.0	2.5	**81.7**	**3.60**
	0.1 (10x LOQ)	79.3	1.4		
(S)-RH-150721	0.01 (LOQ)	83.1	2.7	**81.5**	**2.94**
	0.1 (10x LOQ)	80.0	1.6		
RH-150721 sum	0.01 (LOQ)	83.6	2.4	**81.6**	**3.21**
	0.1 (10x LOQ)	79.6	1.5		
RH-150721: qualifier transition 318 → 186.9					

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
(R)-RH-150721	0.01 (LOQ)	82.7	2.3	**81.5**	**2.42**
	0.1 (10x LOQ)	80.4	1.6		
(S)-RH-150721	0.01 (LOQ)	90.0	3.3	**88.2**	**3.40**
	0.1 (10x LOQ)	86.4	2.0		
RH-150721 sum	0.01 (LOQ)	86.3	2.8	**84.9**	**2.90**
	0.1 (10x LOQ)	83.4	1.8		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	93.6	1.6	**86.5**	**8.8**
	0.1 (10x LOQ)	79.5	2.5		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.0	0.8	**86.1**	**8.6**
	0.1 (10x LOQ)	79.3	3.3		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	95.7	1.1	**88.7**	**8.5**
	0.1 (10x LOQ)	81.8	2.8		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	99.3	1.4	**89.1**	**12.2**
	0.1 (10x LOQ)	78.9	2.8		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	78.7	1.0	**78.5**	**1.6**
	0.1 (10x LOQ)	78.2	2.1		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	82.9	3.9	**77.9**	**7.4**
	0.1 (10x LOQ)	73.0	1.9		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	74.4	3.3	**75.5**	**5.73**
	0.1 (10x LOQ)	76.6	7.5		
RH-129151 (B)	0.01 (LOQ)	75.6	2.6	**75.1**	**6.10**
	0.1 (10x LOQ)	74.7	8.8		
RH-129151 (sum)	0.01 (LOQ)	75.0	2.9	**75.3**	**5.72**
	0.1 (10x LOQ)	75.6	8.0		

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	71.4	1.8	**73.4**	**5.86**
	0.1 (10x LOQ)	75.4	7.2		
RH-129151 (B)	0.01 (LOQ)	70.6	0.5	**71.4**	**6.29**
	0.1 (10x LOQ)	72.3	9.1		
RH-159151 (sum)	0.01 (LOQ)	71.0	0.9	**72.4**	**5.94**
	0.1 (10x LOQ)	73.0	8.2		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	77.4	4.7	**77.5**	**3.79**
	0.1 (10x LOQ)	77.5	3.2		
(S)-RH-141288	0.01 (LOQ)	78.1	5.70	**78.0**	**3.93**
	0.1 (10x LOQ)	77.9	1.6		
RH-141288 (sum)	0.01 (LOQ)	77.8	4.2	**77.7**	**3.17**
	0.1 (10x LOQ)	77.7	2.2		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	72.5	4.5	**74.6**	**4.38**
	0.1 (10x LOQ)	76.7	1.9		
(S)-RH-141288	0.01 (LOQ)	82.9	3.3	**81.2**	**3.40**
	0.1 (10x LOQ)	79.6	2.0		
RH-141288 (sum)	0.01 (LOQ)	77.5	2.7	**77.8**	**2.29**
	0.1 (10x LOQ)	78.1	2.0		

Table A 154: Recovery results in onion

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	84.2	7.7	**83.1**	**5.46**
	0.1 (10x LOQ)	82.0	1.4		
(S)-Zoxamide	0.01 (LOQ)	85.2	7.6	**83.7**	**5.88**
	0.1 (10x LOQ)	82.1	3.2		
Zoxamide sum	0.01 (LOQ)	84.7	7.1	**83.4**	**5.32**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	82.0	2.3		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	83.1	5.2	**82.6**	**3.79**
	0.1 (10x LOQ)	82.1	2.1		
(S)-Zoxamide	0.01 (LOQ)	82.8	5.6	**82.1**	**3.97**
	0.1 (10x LOQ)	81.5	1.5		
Zoxamide sum	0.01 (LOQ)	82.9	5.4	**82.4**	**3.85**
	0.1 (10x LOQ)	81.8	1.8		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	81.7	6.3	**84.3**	**5.5**
	0.1 (10x LOQ)	87.0	2.2		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	92.6	4.9	**90.5**	**4.8**
	0.1 (10x LOQ)	88.3	3.6		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	74.1	10.8	**85.2**	**15.2**
	0.1 (10x LOQ)	96.3	2.6		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	76.1	5.1	**85.3**	**12.0**
	0.1 (10x LOQ)	94.5	3.0		

Table A 155: Recovery results tomato fruit

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	103.0	5.8	**98.3**	**6.46**
	0.1 (10x LOQ)	93.7	1.5		
(S)-Zoxamide	0.01 (LOQ)	104.1	6.6	**98.2**	**7.91**
	0.1 (10x LOQ)	92.3	1.3		
Zoxamide sum	0.01 (LOQ)	103.5	5.8	**98.3**	**7.01**
	0.1 (10x LOQ)	93.0	1.4		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	102.6	6.4	**97.8**	**6.94**
	0.1 (10x LOQ)	92.9	1.7		
(S)-Zoxamide	0.01 (LOQ)	105.9	4.6	**98.2**	**8.94**
	0.1 (10x LOQ)	90.5	1.3		
Zoxamide sum	0.01 (LOQ)	104.3	5.3	**91.7**	**1.4**
	0.1 (10x LOQ)	97.7	1.3		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	97.0	3.9	**96.8**	**2.89**
	0.1 (10x LOQ)	96.6	1.9		
(S)-RH-150721	0.01 (LOQ)	106.3	0.9	**106.2**	**1.74**
	0.1 (10x LOQ)	106.0	2.5		
RH-150721 sum	0.01 (LOQ)	101.6	2.0	**101.4**	**1.69**
	0.1 (10x LOQ)	101.3	1.5		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	99.4	3.7	**96.5**	**4.48**
	0.1 (10x LOQ)	93.6	3.0		
(S)-RH-150721	0.01 (LOQ)	93.6	3.9	**101.1**	**8.26**
	0.1 (10x LOQ)	108.6	1.8		
RH-150721 sum	0.01 (LOQ)	96.5	3.4	**98.8**	**3.62**
	0.1 (10x LOQ)	101.0	2.2		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	86.5	2.9	**85.7**	**2.3**
	0.1 (10x LOQ)	85.0	1.4		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	84.5	5.1	**86.3**	**4.6**
	0.1 (10x LOQ)	88.0	3.5		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	75.9	4.9	**82.0**	**8.6**
	0.1 (10x LOQ)	88.2	1.8		
RH-24549: qualifier transition 202.9672 → 158.9774					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-24549	0.01 (LOQ)	80.9	2.0	**82.8**	**2.9**
	0.1 (10x LOQ)	84.7	1.2		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	73.0	3.0	**79.9**	**9.3**
	0.1 (10x LOQ)	86.8	1.1		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	78.6	6.6	**80.9**	**5.5**
	0.1 (10x LOQ)	83.2	2.6		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	75.2	5.9	**73.3**	**6.31**
	0.1 (10x LOQ)	70.7	5.5		
RH-129151 (B)	0.01 (LOQ)	75.6	11.1	**73.6**	**8.75**
	0.1 (10x LOQ)	71.6	5.1		
RH-129151 (sum)	0.01 (LOQ)	75.4	6.2	**73.3**	**6.24**
	0.1 (10x LOQ)	71.2	5.2		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	74.6	8.2	**73.1**	**6.80**
	0.1 (10x LOQ)	71.6	5.0		
RH-129151 (B)	0.01 (LOQ)	76.4	8.0	**74.4**	**6.9**
	0.1 (10x LOQ)	75.5	4.9		
RH-159151 (sum)	0.01 (LOQ)	75.5	8.0	**73.8**	**6.82**
	0.1 (10x LOQ)	72.0	4.9		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	91.3	3.6	**92.2**	**2.81**
	0.1 (10x LOQ)	93.0	1.6		
(S)-RH-141288	0.01 (LOQ)	90.0	5.5	**91.5**	**4.33**
	0.1 (10x LOQ)	93.1	2.4		
RH-141288 (sum)	0.01 (LOQ)	90.7	4.5	**91.9**	**3.44**
	0.1 (10xLOQ)	93.0	1.6		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	87.5	3.4	**90.8**	**4.63**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	94.2	1.5		
(S)-RH-141288	0.01 (LOQ)	89.5	4.8	**90.6**	**3.65**
	0.1 (10x LOQ)	91.7	2.1		
RH-141288 (sum)	0.01 (LOQ)	88.5	4.0	**90.7**	**3.90**
	0.1 (10x LOQ)	93.0	1.8		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	98.1	3.0	**96.5**	**3.13**
	0.1 (10x LOQ)	95.0	2.5		
(S)-RH-127450	0.01 (LOQ)	95.4	2.8	**95.0**	**2.30**
	0.1 (10x LOQ)	96.6	1.9		
RH-127450 (sum)	0.01 (LOQ)	96.8	2.5	**95.8**	**2.46**
	0.1 (10x LOQ)	94.8	2.2		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	99.0	3.0	**97.0**	**3.27**
	0.1 (10x LOQ)	95.0	2.0		
(S)-RH-127450	0.01 (LOQ)	94.4	3.8	**94.3**	**2.79**
	0.1 (10x LOQ)	94.2	1.7		
RH-127450 (sum)	0.01 (LOQ)	96.7	3.1	**95.7**	**2.67**
	0.1 (10x LOQ)	94.6	1.8		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	81.2	5.5	**84.7**	**5.8**
	0.1 (10x LOQ)	88.3	1.9		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	78.8	4.0	**85.7**	**8.8**
	0.1 (10x LOQ)	92.5	1.2		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	77.1	4.8	**80.6**	**5.5**
	0.1 (10x LOQ)	84.0	0.9		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	76.5	3.8	**78.1**	**3.5**
	0.1 (10x LOQ)	79.7	1.6		

Table A 156: Recovery results in canned tomatoes

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	95.1	1.8	**95.0**	**2.99**
	0.1 (10x LOQ)	94.8	4.1		
(S)-Zoxamide	0.01 (LOQ)	91.2	3.6	**92.3**	**3.93**
	0.1 (10x LOQ)	93.5	4.2		
Zoxamide sum	0.01 (LOQ)	93.2	2.2	**93.6**	**3.19**
	0.1 (10x LOQ)	94.1	4.1		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	93.5	2.8	**94.1**	**3.77**
	0.1 (10x LOQ)	94.7	4.8		
(S)-Zoxamide	0.01 (LOQ)	92.3	2.2	**93.2**	**3.26**
	0.1 (10x LOQ)	94.1	4.0		
Zoxamide sum	0.01 (LOQ)	92.9	2.5	**93.7**	**3.48**
	0.1 (10x LOQ)	94.4	4.4		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	105.6	2.2	**105.3**	**2.41**
	0.1 (10x LOQ)	105.0	2.8		
(S)-RH-150721	0.01 (LOQ)	107.5	2.3	**108.3**	**3.19**
	0.1 (10x LOQ)	109.1	4.0		
RH-150721 sum	0.01 (LOQ)	106.7	1.4	**106.8**	**2.43**
	0.1 (10x LOQ)	107.0	3.4		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	99.3	2.7	**102.3**	**3.83**
	0.1 (10x LOQ)	105.2	2.2		
(S)-RH-150721	0.01 (LOQ)	110.5	0.8	**109.8**	**3.022**
	0.1 (10x LOQ)	109.2	4.4		
RH-150721 sum	0.01 (LOQ)	104.8	1.4	**106.0**	**2.53**
	0.1 (10x LOQ)	107.2	3.0		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	86.8	1.5	**86.8**	**4.00**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	86.8	5.8		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	79.1	2.3	**84.1**	**7.33**
	0.1 (10x LOQ)	89.1	4.9		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	86.7	0.6	**89.1**	**3.89**
	0.1 (10x LOQ)	91.4	3.9		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.8	2.0	**91.2**	**4.28**
	0.1 (10xLOQ)	88.5	4.1		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	91.7	1.8	**91.6**	**3.21**
	0.1 (10x LOQ)	91.4	4.5		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	88.9	2.1	**88.4**	**3.32**
	0.1 (10x LOQ)	88.0	4.5		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	72.4	9.0	**71.4**	**7.7**
	0.1 (10x LOQ)	70.3	6.8		
RH-129151 (B)	0.01 (LOQ)	75.5	10.0	**72.9**	**8.9**
	0.1 (10x LOQ)	70.2	6.4		
RH-129151 (sum)	0.01 (LOQ)	73.9	9.4	**72.1**	**8.51**
	0.1 (10x LOQ)	70.2	6.6		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	786.2	8.8	**73.9**	**7.9**
	0.1 (10x LOQ)	71.6	6.0		
RH-129151 (B)	0.01 (LOQ)	75.0	8.0	**73.3**	**6.9**
	0.1 (10x LOQ)	71.6	5.4		
RH-159151 (sum)	0.01 (LOQ)	75.6	8.4	**73.6**	**7.39**
	0.1 (10x LOQ)	71.6	5.6		
RH-141288: quantifier transition 320 → 206					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
(R)-RH-141288	0.01 (LOQ)	95.8	1.1	**93.3**	**4.21**
	0.1 (10x LOQ)	90.9	4.7		
(S)-RH-141288	0.01 (LOQ)	95.5	1.7	**91.6**	**4.73**
	0.1 (10x LOQ)	87.7	1.4		
RH-141288 (sum)	0.01 (LOQ)	95.6	1.2	**92.5**	**3.99**
	0.1 (10x LOQ)	89.3	2.3		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	95.1	3.8	**91.7**	**5.29**
	0.1 (10x LOQ)	88.3	3.8		
(S)-RH-141288	0.01 (LOQ)	99.9	4.0	**94.3**	**7.01**
	0.1 (10x LOQ)	88.6	1.6		
RH-141288 (sum)	0.01 (LOQ)	97.4	3.1	**93.0**	**5.62**
	0.1 (10x LOQ)	88.5	1.5		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	98.5	3.1	**99.0**	**3.51**
	0.1 (10x LOQ)	99.5	4.1		
(S)-RH-127450	0.01 (LOQ)	91.5	1.2	**93.0**	**3.86**
	0.1 (10x LOQ)	84.5	5.0		
RH-127450 (sum)	0.01 (LOQ)	95.0	1.7	**96.0**	**3.45**
	0.1 (10x LOQ)	97.0	4.6		
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	95.9	3.3	**97.7**	**3.85**
	0.1 (10x LOQ)	99.6	3.8		
(S)-RH-127450	0.01 (LOQ)	93.5	1.80	**94.0**	**3.86**
	0.1 (10x LOQ)	94.4	5.4		
RH-127450 (sum)	0.01 (LOQ)	94.6	2.1	**95.8**	**3.64**
	0.1 (10x LOQ)	97.0	4.6		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	94.0	1.8	**93.5**	**3.17**
	0.1 (10x LOQ)	93.1	4.4		
RH-149736: qualifier transition 219.9927 → 158.9761					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-149736	0.01 (LOQ)	88.3	2.8	**90.0**	**3.61**
	0.1 (10x LOQ)	91.8	3.5		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	84.9	4.8	**88.5**	**5.73**
	0.1 (10x LOQ)	92.2	3.1		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	103.7	8.7	**97.3**	**9.73**
	0.1 (10x LOQ)	90.8	4.6		

Table A 157: Recovery results in cucumber

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
Zoxamdie: quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	82.6	4.7	**83.0**	**3.40**
	0.1 (10x LOQ)	83.4	1.9		
(S)-Zoxamide	0.01 (LOQ)	84.4	1.1	**84.4**	**1.29**
	0.1 (10x LOQ)	84.3	1.6		
Zoxamide sum	0.01 (LOQ)	83.5	2.8	**83.7**	**2.17**
	0.1 (10x LOQ)	83.9	1.5		
Zoxamide: qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	87.6	3.0	**85.6**	**3.27**
	0.1 (10x LOQ)	83.6	1.3		
(S)-Zoxamide	0.01 (LOQ)	87.8	1.8	**86.2**	**2.67**
	0.1 (10x LOQ)	84.6	2.0		
Zoxamide sum	0.01 (LOQ)	87.7	2.2	**85.9**	**2.81**
	0.1 (10x LOQ)	84.1	1.4		
RH-150721: quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	99.6	5.7	**100.7**	**5.54**
	0.1 (10x LOQ)	101.9	5.8		
(S)-RH-150721	0.01 (LOQ)	83.7	2.5	**92.2**	**9.97**
	0.1 (10x LOQ)	100.7	2.2		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-150721 sum	0.01 (LOQ)	91.6	3.0	**96.5**	**5.99**
	0.1 (10x LOQ)	101.3	3.0		
RH-150721: qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	101.6	4.1	**102.3**	**4.60**
	0.1 (10x LOQ)	103.0	5.4		
(S)-RH-150721	0.01 (LOQ)	86.6	4.11.2	**93.6**	**8.33**
	0.1 (10x LOQ)	100.7			
RH-150721 sum	0.01 (LOQ)	94.2	3.3	**98.0**	**5.15**
	0.1 (10x LOQ)	101.8	3.2		
RH-139432: quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	97.0	3.6	**94.3**	**5.9**
	0.1 (10x LOQ)	91.6	6.7		
RH-139432: qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	82.0	6.3	**88.4**	**9.1**
	0.1 (10x LOQ)	94.7	4.3		
RH-24549: quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	85.2	5.1	**89.0**	**6.8**
	0.1 (10x LOQ)	92.9	5.5		
RH-24549: qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	91.7	3.6	**91.4**	**3.9**
	0.1 (10x LOQ)	91.0	4.6		
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	91.8	3.6	**92.2**	**4.4**
	0.1 (10x LOQ)	92.6	5.4		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	93.7	5.3	**90.9**	**6.3**
	0.1 (10x LOQ)	88.2	6.3		
RH-129151: quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	90.2	11.7	**87.2**	**10.38**
	0.1 (10x LOQ)	84.2	8.3		
RH-129151 (B)	0.01 (LOQ)	96.9	9.4	**90.6**	**10.74**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	84.2	6.4		
RH-129151 (sum)	0.01 (LOQ)	93.6	10.5	**88.9**	**10.34**
	0.1 (10x LOQ)	84.2	7.4		
RH-129151: qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	94.2	10.0	**89.2**	**9.35**
	0.1 (10x LOQ)	86.0	7.9		
RH-129151 (B)	0.01 (LOQ)	100.5	6.5	**94.5**	**9.04**
	0.1 (10x LOQ)	88.5	6.3		
RH-159151 (sum)	0.01 (LOQ)	96.5	8.1	**91.9**	**8.94**
	0.1 (10x LOQ)	87.3	7.0		
RH-141288: quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	80.7	4.6	**83.2**	**4.39**
	0.1 (10x LOQ)	85.6	1.2		
(S)-RH-141288	0.01 (LOQ)	81.8	2.8	**82.7**	**2.68**
	0.1 (10x LOQ)	83.6	2.2		
RH-141288 (sum)	0.01 (LOQ)	81.2	3.2	**82.9**	**3.22**
	0.1 (10x LOQ)	84.7	1.7		
RH-141288: qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	76.3	1.3	**80.0**	**5.16**
	0.1 (10x LOQ)	83.7	2.0		
(S)-RH-141288	0.01 (LOQ)	75.6	3.1	**78.5**	**4.85**
	0.1 (10x LOQ)	81.4	3.0		
RH-141288 (sum)	0.01 (LOQ)	75.9	2.1	**79.3**	**4.91**
	0.1 (10x LOQ)	82.6	2.4		
RH-127450: quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	74.7	3.4	**77.7**	**4.76**
	0.1 (10x LOQ)	80.7	1.8		
(S)-RH-127450	0.01 (LOQ)	81.3	1.4	**82.7**	**2.56**
	0.1 (10x LOQ)	84.0	2.4		
RH-127450 (sum)	0.01 (LOQ)	78.0	2.2	**80.2**	**3.47**
	0.1 (10x LOQ)	82.4	1.9		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-127450: qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	75.7	2.0	**78.5**	**4.21**
	0.1 (10x LOQ)	81.4	1.7		
(S)-RH-127450	0.01 (LOQ)	85.2	3.4	**85.1**	**2.99**
	0.1 (10x LOQ)	85.0	2.9		
RH-127450 (sum)	0.01 (LOQ)	80.4	2.3	**81.8**	**2.77**
	0.1 (10x LOQ)	83.2	2.2		
RH-149736: quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	97.4	3.8	**98.4**	**3.9**
	0.1 (10xLOQ)	99.4	4.1		
RH-149736: qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	102.2	3.2	**101.3**	**3.6**
	0.1 (10x LOQ)	100.3	4.1		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	89.3	11.1	**89.4**	**8.3**
	0.1 (10x LOQ)	89.5	5.5		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	88.4	11.8	**89.0**	**8.4**
	0.1 (10x LOQ)	89.6	4.7		

Table A 158: Recovery results in grape fruits (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	106.5	1.0	**105.8**	**1.8**
	0.1 (10x LOQ)	105.0	2.2		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	87.2	1.0	**94.8**	**8.5**
	0.1 (10x LOQ)	102.4	1.1		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	109.4	1.7	**109.4**	**1.3**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	109.4	1.1		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	98.0	4.0	**102.0**	**5.1**
	0.1 (10x LOQ)	106.1	2.2		

Table A 159: Recovery results in grape juice (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	106.4	2.2	**103.5**	**3.5**
	0.1 (10x LOQ)	100.6	1.7		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	104.0	2.9	**103.4**	**2.2**
	0.1 (10x LOQ)	102.7	1.3		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	108.0	3.3	**107.5**	**2.3**
	0.1 (1x LOQ)	107.0	0.9		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	107.5	2.0	**107.0**	**1.7**
	0.1 (10x LOQ)	106.5	1.3		

Table A 160: Recovery results in wine (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	94.6	4.3	**92.3**	**4.0**
	0.1 (10x LOQ)	90.1	1.0		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	81.8	3.8	**84.7**	**4.5**
	0.1 (10x LOQ)	87.6	1.7		
RH-149737: quantifier transition 187.9675 (SIM)					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-149737	0.01 (LOQ)	106.8	2.1	**100.8**	**6.6**
	0.1 (10x LOQ)	94.8	2.3		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	107.1	2.1	**101.5**	**6.0**
	0.1 (10x LOQ)	96.0	1.1		

Table A 161: Recovery results in raisins (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	72.5	0.4	**72.1**	**0.8**
	0.1 (10x LOQ)	71.8	0.8		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	74.9	2.9	**72.6**	**3.95**
	0.1 (10x LOQ)	70.3	1.3		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	79.1	3.6	**92.8**	**15.76**
	0.1 (10x LOQ)	106.5	2.0		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	73.8	4.3	**86.6**	**15.97**
	0.1 (10x LOQ)	99.8	0.5		

Table A 162: Recovery results in potato tubers (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	93.3	6.5	**91.0**	**5.3**
	0.1 (10x LOQ)	88.6	1.5		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	110.7	13.8	**100.7**	**14.9**
	0.1 (10x LOQ)	90.7	5.3		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	97.1	19.3	**92.6**	**14.6**
	0.1 (10x LOQ)	88.1	2.6		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	109.0	3.6	**99.3**	**10.7**
	0.1 (10x LOQ)	89.5	1.8		

Table A 163: Recovery results in potato flakes (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	99.2	3.3	**97.1**	**3.37**
	0.1 (10xLOQ)	95.0	1.7		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	102.2	4.3	**99.8**	**4.31**
	0.1 (10x LOQ)	97.3	2.8		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	79.3	7.0	**77.4**	**5.46**
	0.1 (10x LOQ)	75.6	1.7		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	73.2	3.0	**71.7**	**3.23**
	0.1 (10x LOQ)	70.3	2.0		

Table A 164: Recovery results in fried potatoes (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	86.9	2.3	**85.1**	**2.8**
	0.1 (10x LOQ)	83.3	0.5		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	95.1	1.4	**87.9**	**8.8**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
	0.1 (10x LOQ)	80.7	2.8		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	71.9	3.9	**71.3**	**2.8**
	0.1 (10x LOQ)	70.7	0.1		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	76.6	4.1	**73.3**	**5.5**
	0.1 (10x LOQ)	70.1	1.0		

Table A 165: Recovery results in tomato fruits (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	72.6	1.6	**81.5**	**11.7**
	0.1 (10x LOQ)	90.4	2.7		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	81.8	1.9	**84.7**	**3.9**
	0.1 (10x LOQ)	87.6	1.6		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	75.8	2.8	**87.4**	**14.3**
	0.1 (10x LOQ)	99.1	2.5		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	74.3	3.9	**86.8**	**15.5**
	0.1 (10x LOQ)	99.4	2.0		

Table A 166: Recovery results in canned tomatoes (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	104.7	3.2	**104.0**	**2.41**
	0.1 (10x LOQ)	103.3	1.3		
RH-141452: qualifier transition 218.9621 → 144.9617					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452	0.01 (LOQ)	106.4	2.2	**102.6**	**4.22**
	0.1 (10x LOQ)	98.9	1.4		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	109.1	0.8	**108.4**	**0.92**
	0.1 (10x LOQ)	107.8	0.6		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	102.5	4.0	**105.7**	**4.21**
	0.1 (10x LOQ)	108.9	1.4		

Table A 167: Recovery results in cucumber (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	82.6	14.0	**82.3**	**11.2**
	0.1 (10x LOQ)	82.0	9.3		
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	101.1	9.8	**90.2**	**15.9**
	0.1 (10x LOQ)	79.4	10.4		
RH-149737: quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	83.2	19.8	**85.2**	**14.0**
	0.1 (10x LOQ)	87.2	7.2		
RH-149737: qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	92.4	17.9	**90.7**	**13.1**
	0.1 (10x LOQ)	89.0	6.9		

Table A 168: Recovery results in onion (conjugates)

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	83.2	3.6	**82.2**	**3.3**
	0.1 (10x LOQ)	81.1	2.8		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	97.4	5.5	**89.3**	**10.6**
	0.1 (10xL OQ)	81.2	2.9		
RH-141455: quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	87.0	3.8	**79.7**	**10.2**
	0.1 (10x LOQ)	72.3	1.5		
RH-141455: qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	94.8	4.6	**82.9**	**15.5**
	0.1 (10x LOQ)	71.7	1.7		

Accuracy and repeatability/precision

Accuracy and precision were verified by means of recovery test carried out at 2 spiking levels:
- 0.01 mg/kg (0.005 mg/kg for each single enantiomer) corresponding to the target LOQ
- 0.1 mg/kg (0.005 mg/kg for each single enantiomer) corresponding to 10 x LOQ

The mean recoveries per spiking level for both primary and confirmatory transition fulfil the guideline requirements (mean recovery per level in the range 70-110% and RSD% per level < 20%).

Linearity

Linearity of response was verified as follows with matrix-matched standard solutions.

- 5 points calibration curves.
- Calibration ranges of 1 - 50 µg/L on final extract
  Exceptions: for wine and grape juice samples, nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flake samples the lowest concentration was 0.7 µg/L (corresponding to 2.8 µg/kg in the sample).
- Range of 3 - 160 mg/kg per sample, corresponding to 30% LOQ – 50 x LOQ.
- For analytes with a chiral centre: Considering the sum of 2 enantiomers.
- R^2 > values 0.99.
- Linearity always checked for 2 MRM (Multiple Reaction Monitoring) transitions in case of Triple quadrupole mass analysis or for the 2 high resolution ions in case of HRMS-Orbitrap analysis.

Limit of quantification (all analyte/matrix)

- 0.01 mg/kg. For analytes with a chiral centre this limit is referred to the sum of the 2 enantiomers (i.e. the LOQ for each enantiomer is 0.005 mg/kg)

Limit of detection (all analyte/matrix)

- 1 µg/L / 0.003 mg/kg on sample / 30% of LOQ (0.01 mg/kg)
- Signal to noise ratio measured at LOD for all analyte/matrix was always higher than 3
- For analytes with a chiral centre this limit is referred to the sum of the 2 enantiomers (i.e. the LOD for each enantiomer is 0.0015 mg/kg).

Matrix effects

All calibration curves, for all matrices/analyte were prepared using matrix matched analytical standards. Blank sample extracts, prepared with the same procedure applied on spiked samples were used to dilute stock solutions. Using this approach, the matrix effect on all samples analysed was nullified.

Specificity

The methods are highly specific with characteristic Rf-values and the measurement of 2 MRM (Multiple Reaction Monitoring) transitions in case of Triple quadrupole mass analysis or 2 high resolution ions in case of HRMS-Orbitrap analysis per analyte.

For all analytes in all matrices the blank sample signal (for both for the primary and the confirmatory detection) was always < 30% LOQ, in most cases the signal in the blank samples was zero (i.e. no detectable/quantifiable at analyte retention time).

Stability of sample extracts

The stability of the analytes in the final extracts stored at around 4°C was confirmed by recovery experiments for a storage period of 3 days, corresponding to the maximum period of time elapsed between sample extraction and analysis. The stability of the extracts was considered proven if the difference between the instrument response for a freshly prepared spiked samples compared with the stored ones was ≤ ±20%.

The metabolite RH-129151 was the only analyte not stable in the final extract. Therefore, the analysis of this analyte in all samples needs to be and was always carried out immediately after extraction (analytical sequences in this study completed within 8 hours after sample extraction).

Table A 169: Characteristics for the analytical method used for validation of zoxamide and its metabolites in plant matrices

	Zoxamide (sum, R/S), RH-150721 (sum, R/S), RH-129151 (sum, R/S), RH-141288 (sum, R/S), RH-127450 (sum, R/S), RH-149736, RH-149737, RH-24549, RH-139432, RH-141452, RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix-matched standard calibration. 5-point calibration; r^2 = >0.99; linear Calibration data and calibration line equation presented in the study report.
Calibration range	1-50 µg/L on final extract Exceptions: for wine and grape juice samples nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flakes the lowest concentration was 0.7µg/L (corresponding to a concentration of 2.8 µg/kg and 3-160 mg/kg in the sample)
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.01 mg/kg For analytes with a chiral centre this limit is referred to the sum of 2 enantiomers (i.e. the LOQ for each enantiomer is 0.005 mg/kg). LOD: 0.003 mg/kg for sample 0.0015 mg/kg for each enantiomer with a chiral centre

The following figures show example chromatograms.

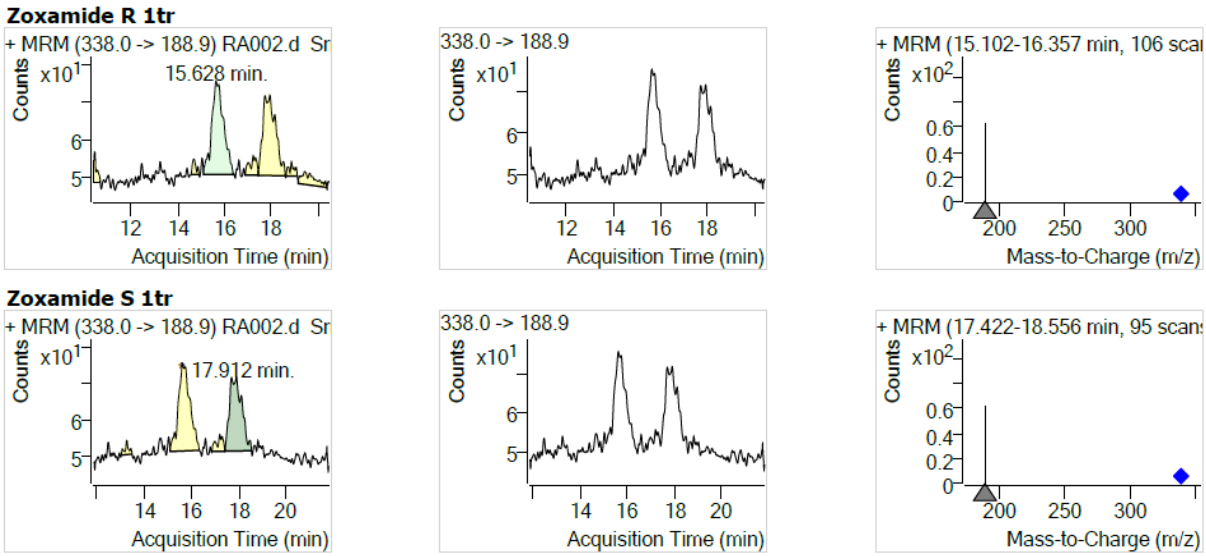


Figure A 64: Chromatogram of Zoxamide (R/S)

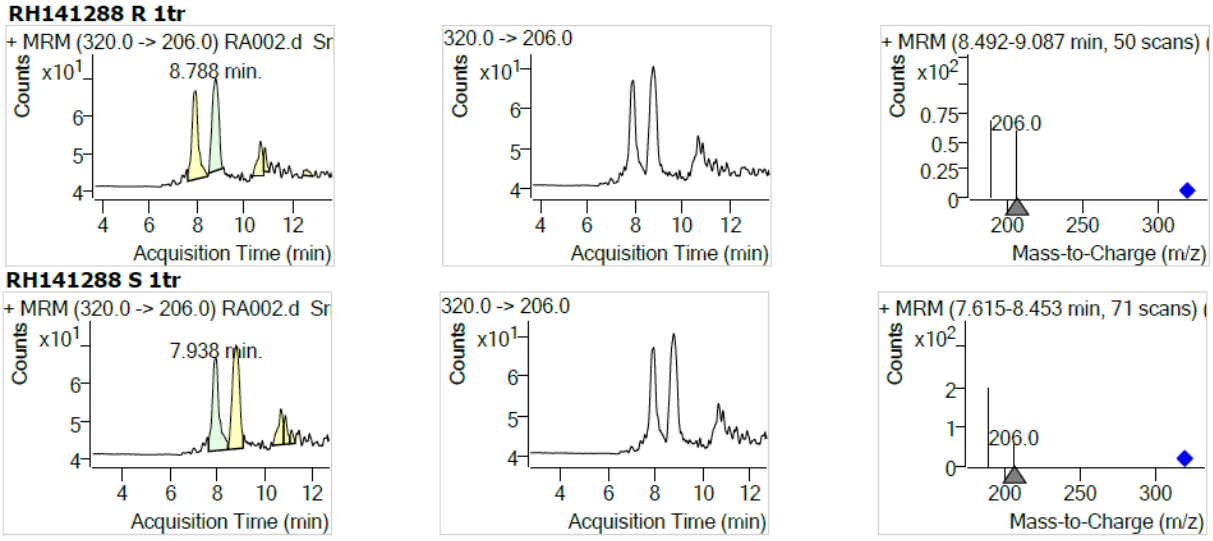


Figure A 65: Chromatogram of RH-141288 (R/S)

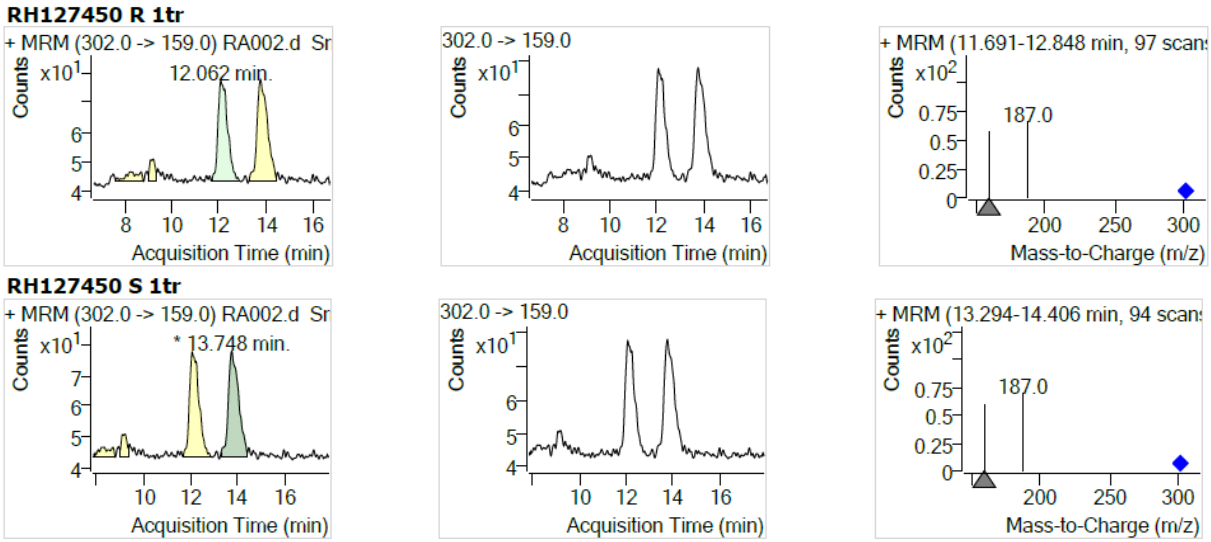


Figure A 66: Chromatogram of RH-127450 (R/S)

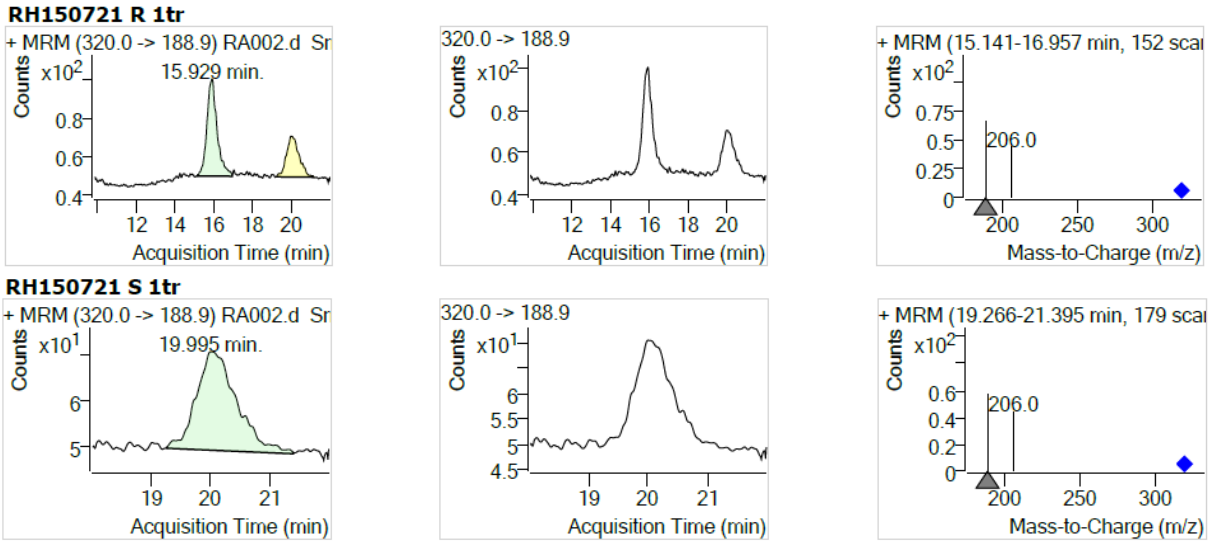


Figure A 67: Chromatogram of RH-150721 (R/S)

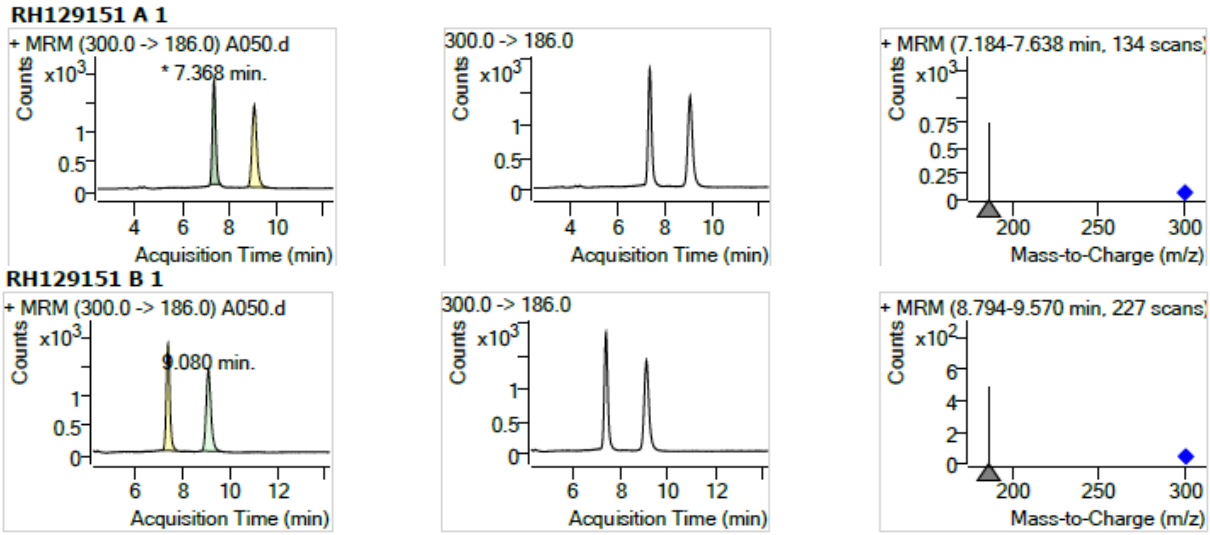


Figure A 68: Chromatogram of RH-129151 (A/B)

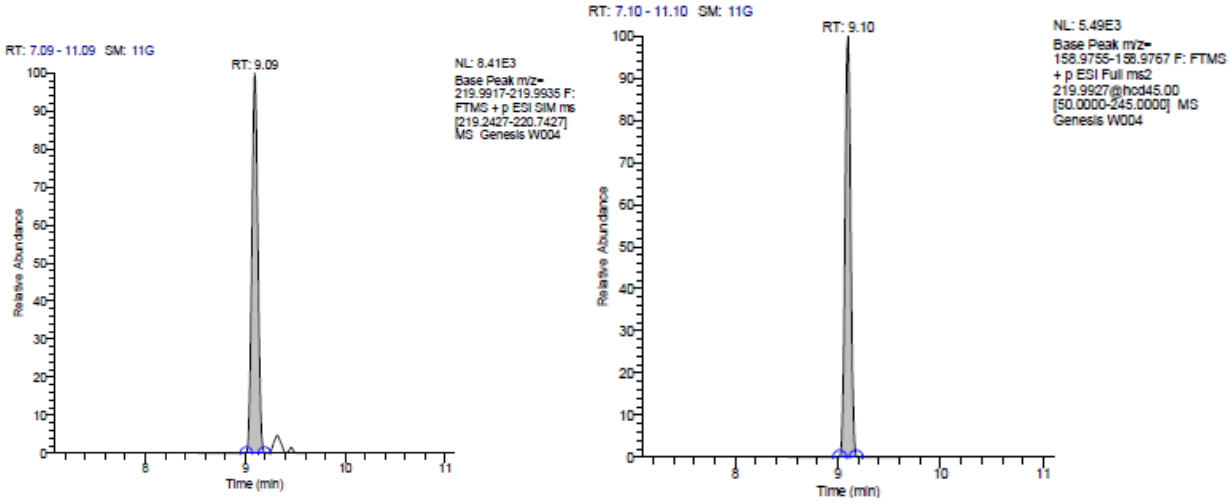


Figure A 69: Chromatogram of RH-149736

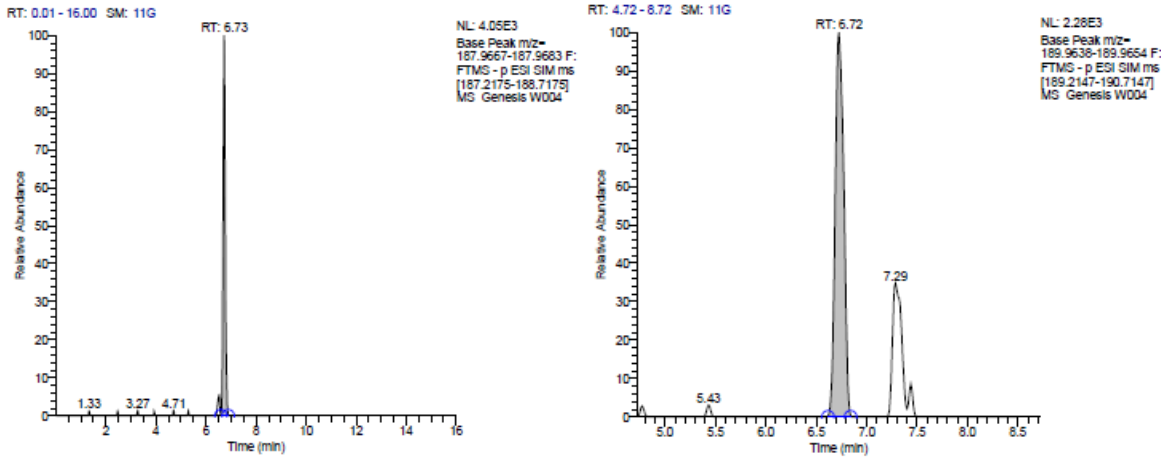


Figure A 70: Chromatogram of RH-149737

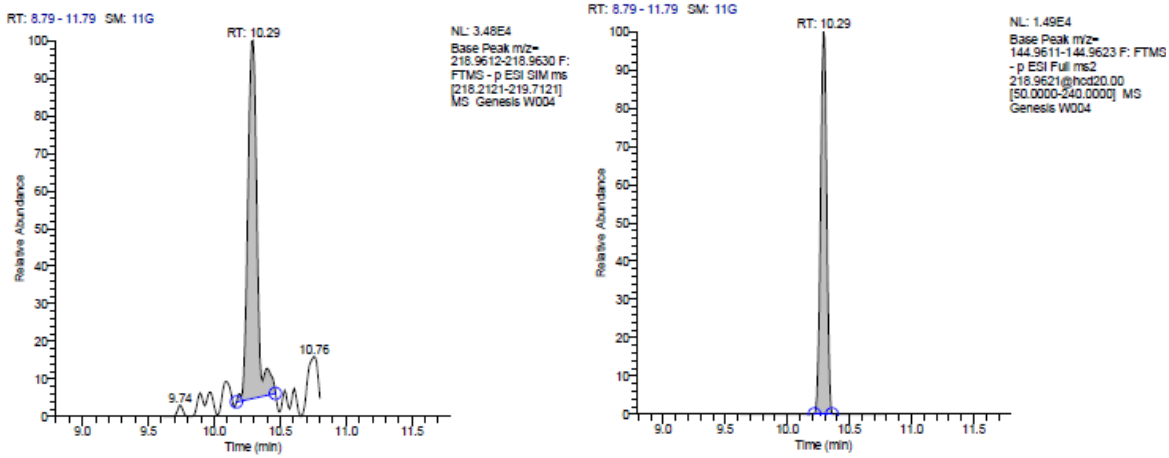


Figure A 71: Chromatogram of RH-141452

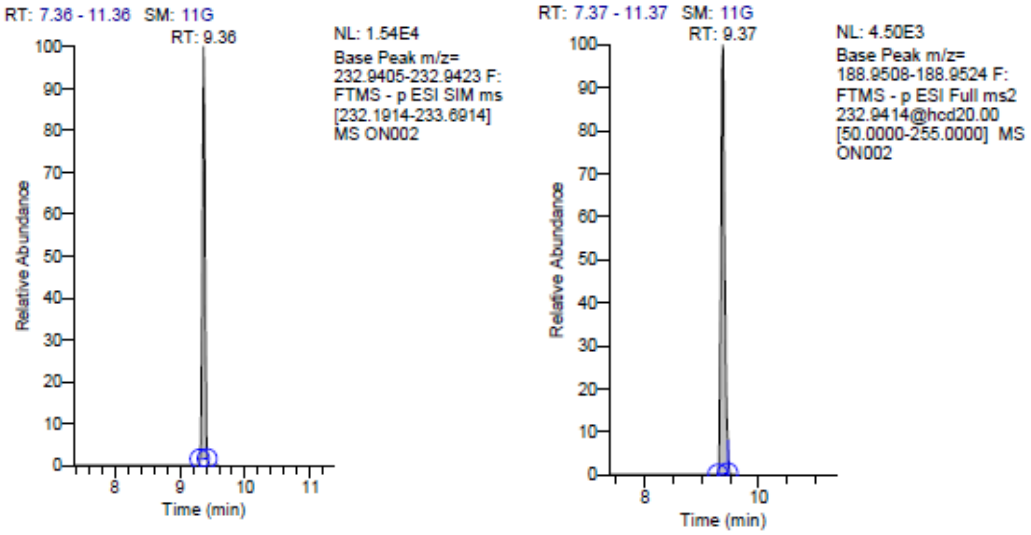


Figure A 72: Chromatogram of RH-141455

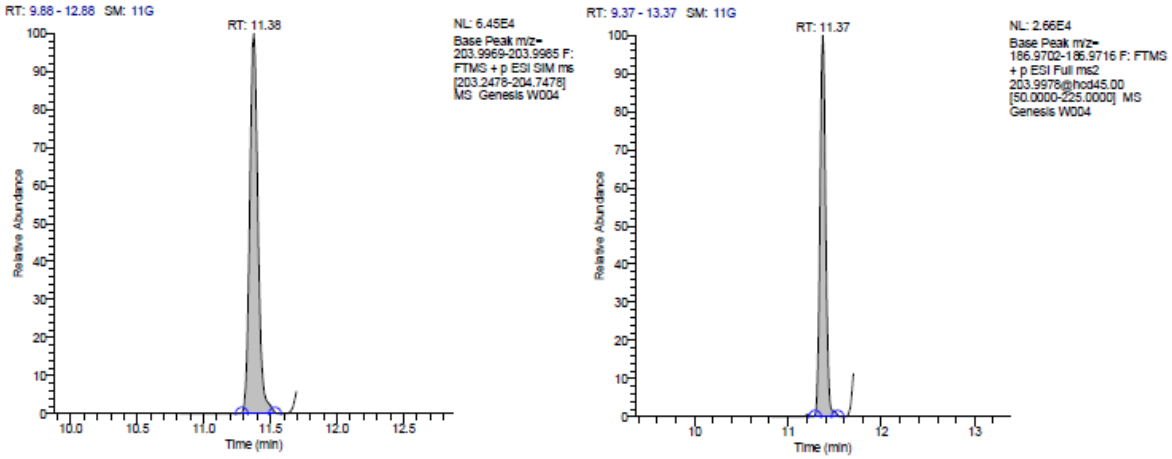


Figure A 73: Chromatogram of RH-139432

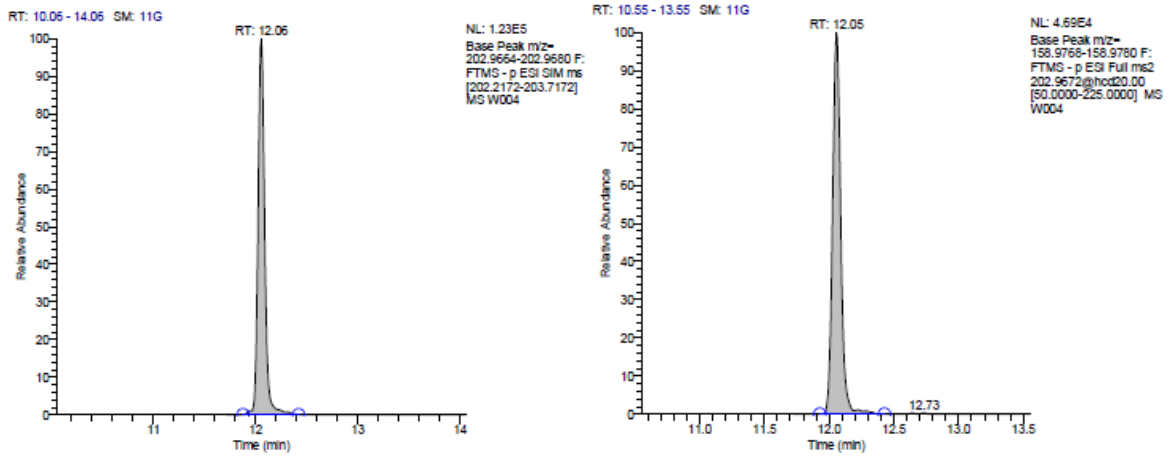


Figure A 74: Chromatogram of RH-24549

Extraction efficiency

The extraction efficiency of the final analytical method has been studied in compliance with SANTE 2017/10632 rev. 3 by comparing the recoveries obtained applying the method under validation with the recoveries of the methods used in the plant metabolism studies performed with radiolabelled test item – and therefore allowing the bilancing of the analytes. A cross validation approach was applied.

Representative crop samples were sprayed with a mixture containing all the analytes. The sprayed samples were left 3 days at room temperature in daylight. After this period the samples were frozen, homogenized by a vegetable grinder, and analysed with the analytical method under validation. In parallel, aliquots of the same samples were extracted with the methods used in the plant metabolism studies, which are also summarised in the RAR zoxamide (2017). The results obtained with the 2 different extraction procedures were compared to assess the extraction efficiency of the method.

For all 4 matrices tested (grapes berries, potato tuber, tomato fruits and cucumber fruits) 3 mL of a water mixture containing all the analytes to be determined at a concentration of 100 µg/mL (each analyte) was applied. According to SANTE 2017/10632 rev. 3 the extraction efficiency was considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the relevant plant metabolism study differs no more than 30%.

The following table summarises the results of extraction efficiency check:

Table A 170: Extraction efficiency for analytes from RAC samples (grape berries)

Grape berries – Extraction efficiency				
Analyte	**Replicate**	**Analyte peak area**		**Extraction efficiency % (mean value of 2 independent extractions)**
		Metabolism study extraction method	**Analytical method under validation**	
(R)-Zoxamide	1	81852	89214	+2%
	2	91088	86621	
(S)-Zoxamide	1	72308	74843	-2%
	2	76504	70532	
(R)-RH-150721	1	154482	156253	-4%
	2	170255	156819	
(S)-RH-150721	1	250461	247878	-1%
	2	260499	258590	
RH-139432	1	3559481	2565170	-17%
	2	3383644	3222062	
RH-24549	1	4171856	3186616	-13%
	2	4136767	4048628	
RH-141452	1	1379681	1086517	-9%
	2	1373294	1408383	
RH-129151 (A)	1	23402162	20828250	-10%
	2	23665786	21303594	
RH-129151 (B)	1	22193362	20868087	-6%
	2	21979252	20692495	
(R)-RH-141288	1	12505	11221	-12%
	2	12071	10492	
(S)-RH-141288	1	5320	5026	-10%
	2	5438	4699	

RH-149736	1	522164	378331	-14%
	2	493359	493311	
(R)-RH-127450	1	66474	70123	+3%
	2	67799	68275	
(S)-RH-127450	1	87867	97113	0%
	2	93774	85103	
RH-149737	1	262596	240245	+6%
	2	263265	315253	

Table A 171: Extraction efficiency for analytes from RAC samples (potato tubers)

Potato tubers – Extraction efficiency				
Analyte	**Replicate**	**Analyte area**		**Extraction efficiency % (mean value of 2 independent extractions)**
		Metabolism study extraction method	**Analytical method under validation**	
(R)-Zoxamide	1	88075	91964	+5%
	2	89201	94891	
(S)-Zoxamide	1	62899	72442	+6%
	2	73640	71767	
(R)-RH-150721	1	249032	271447	+5%
	2	272127	278019	
(S)-RH-150721	1	293180	309124	+9%
	2	299064	336353	
RH-24549	1	9453938	9607449	-1%
	2	9355099	9044082	
RH-141452	1	4667107	4821012	-1%
	2	4592359	4382744	
RH-141455	1	2386000	2550540	+3%
	2	2365070	2363198	
RH-129151 (A)	1	23046998	21674663	-4%
	2	22223143	21890623	
RH-129151 (B)	1	21528424	20342993	-5%
	2	21282012	20276995	
(R)-RH-141288	1	53869	55259	-8%
	2	69748	58710	
(S)-RH-141288	1	53260	69109	+20%
	2	62614	70394	

Table A 172: Extraction efficiency for analytes from RAC samples (tomato fruits)

Tomato fruits – Extraction efficiency				
Analyte	**Replicate**	**Analyte area**		**Extraction efficiency % (mean value of 2 independent extractions)**
		Metabolism study extraction method	**Analytical method under validation**	
(R)-Zoxamide	1	83905	90994	+4%
	2	91147	90728	

(S)-Zoxamide	1	75763	91580	+15%
	2	84524	93109	
(R)-RH-150721	1	169120	207012	+18%
	2	183471	210100	
(S)-RH-150721	1	284660	335145	+10%
	2	317201	329640	
RH-139432	1	2230571	2018713	-3%
	2	2082695	2148639	
RH-24549	1	2936002	2674100	-2%
	2	2643388	2772934	
RH-141452	1	1902036	1815960	-4%
	2	1911972	1860780	
RH-129151 (A)	1	21991802	21772526	0%
	2	21195613	21630008	
RH-129151 (B)	1	20469934	20107901	0%
	2	20018221	20357183	
(R)-RH-141288	1	6549	7695	+9%
	2	7005	7131	
(S)-RH-141288	1	5430	5841	+1%
	2	5895	5615	
RH-149736	1	610292	586978	-4%
	2	609946	580792	
(R)-RH-127450	1	78125	86430	+2%
	2	87494	83187	
(S)-RH-127450	1	103994	120885	+13%
	2	108706	118719	
RH-149737	1	365765	444948	+29%
	2	364025	494130	

Table A 173: Extraction efficiency for analytes from RAC samples (cucumber fruits)

Cucumber fruits – Extraction efficiency				
Analyte	**Replicate**	**Analyte area**		**Extraction efficiency % (mean value of 2 inde-pendent extractions)**
		Metabolism study extraction method	**Analytical method under validation**	
(R)-Zoxamide	1	136364	116047	-8%
	2	124755	123961	
(S)-Zoxamide	1	135159	126455	+2%
	2	121201	134124	
(R)-RH-150721	1	392693	387673	+7%
	2	359808	419802	
(S)-RH-150721	1	402736	359426	-9%
	2	386068	354656	
RH-139432	1	2137497	2094957	-1%

	2	1962368	1965768	
RH-24549	1	3154266	3062499	-2%
	2	3096751	3049544	
RH-141452	1	1192494	1094435	-4%
	2	1122965	1130272	
RH-129151 (A)	1	20390772	20925150	+2%
	2	20445072	20856380	
RH-129151 (B)	1	18298852	19078967	+2%
	2	18797528	18858549	
(R)-RH-141288	1	8672	10480	+22%
	2	8970	11005	
(S)-RH-141288	1	38991	39812	+12%
	2	34786	42826	
RH-149736	1	324943	299121	-8%
	2	324075	296866	
(R)-RH-127450	1	130607	122719	0%
	2	119007	127396	
(R)-RH-127450	1	185545	169812	+1%
	2	158706	178419	
RH-149737	1	281996	258974	-8%
	2	291635	266754	

Conclusion

A highly specific HPLC-MS/MS method for the determination of zoxamide and its metabolites residues in grape, potato, tomato, cucumber, and onion has been validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) at an LOQ of 0.01 mg/kg (for analytes with a chiral centre this limit is referred to the sum of the 2 enantiomers; i.e. the LOQ for each enantiomer is 0.005 mg/kg). The extraction efficiency of the method has been demonstrated for different crop commodities (acidic, high starch content and watery matrix).

(Sala A. 2020)

A 2.1.2.1.3 Analytical method 3

In the following study the multi-residue method (QuEChERS method) based on Anastassiades (2003) using high performance liquid chromatography (HPLC) with a chiral column and mass-spectrometric (MS-MS) detection has been developed and fully validated according to SANCO/825/00 rev. 8.1 (2010) for the determination of zoxamide in honey.

A 2.1.2.1.3.1 Method validation

~~Comments of zRMS:~~	~~The study and method acceptable.~~ ~~For evaluation please, see section 7 of the present RR.~~

Comments of zRMS:	The study is acceptable.

	4 trials (2 NEU, 2 SEU) were performed. The objective of this study was to determine residues of Zoxamide in honey made of Phacelia flowers after application of GWN-9790EU at a worst-case use pattern of 3 x 180 g a.s./ha in field tunnels as far as possible (BBCH 61-65) to the flowering crop. The employed LC-MS/MS method can be regarded as highly specific. The method was fully validated according to SANCO/3029/99 rev. 4 and SANCO/825/00 guideline for (R)- and (S)-Zoxamide, and Zoxamide (sum). The limit of quantification and limit of detection were set to 0.01 mg/kg and 0.003 mg/kg, respectively for Zoxamide. The validity of the analytical method was proven by analysis of fortified samples (high and low concentration), five replicates each, and two validation blank samples. The recoveries of the validation samples were within the range of 70-110% with an RSD of below 20%. The highest Zoxamide residues in Phacelia honey amounted 0.0784 mg/kg however, mostly were <LOQ.

Reference:	**KCP 5.2/03**
Report	Poráczki, K., 2020: Magnitude of residues of zoxamide in phacelia (*Phacelia tanacetifolia* BENTH.) honey after three applications of GWN-9790EU under semi-field conditions in Northern and Southern Europe Gowan Crop Protection Ltd., UK BioChem agrar, Germany., Report No. 19 48 BTR 0003, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANCO/825/00 rev. 8.1 (2010)
Deviations:	Sampling: No sampling of honey for trial 19BTR0003_T3, the following Specimen could not be generated: 19BTR0003_06-T-A1 19BTR0003_06-T-A2 19BTR0003_06-T-R1 19BTR0003_06-T-R2 19BTR0003_06-T-PD Respective samples could not be generated. Trial 3 was repeated
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of zoxamide (racemate) in honey after field applications of the test item GWN-9790EU to *Phacelia tanacetifolia* under semi-field conditions in Northern and Southern Europe.

The analysis of specimens was conducted by an in-house developed method based on the extraction method of the multi-residue method (QuEChERS method) of Anastassiades (2003) using high performance liquid chromatography (HPLC) on a chiral column and mass-spectrometric (MS-MS) detection at a limit of quantification of 0.01 mg/kg for Zoxamide (racemate) based on the method of Jooß (2013). This method is regarded as highly specific. Zoxamide (racemate) concentrations were determined as sum of both isomer peaks. The reference standards of the two single isomers of the active substance Zoxamide will be used to distinct between the (R)- and the (S)-isomer peaks.

For sample preparation, to 3 g of samples (honey) each 15 mL of water and acetonitrile were added prior to extraction on a Fastprep instrument (3 cycles, 20 s at speed 5 m/s, 15 s pause). Afterwards, about 3.5 g salt mixture (4:1:1:1 magnesium sulfate: sodium chloride: trisodium citrate dihydrate: disodium hydrogen citrate sesquihydrate) were added and the Fastprep extraction was repeated (3 cycles, 20 s at speed 5 m/s,

15 s pause). All samples were centrifuged at 5000 rpm for 5 min. The resulting acetonitrile phases were diluted with water (and blank extract, if necessary) and injected into the HPLC-system. The two enantiomers of zoxamide were separated on a chiral column by high performance liquid chromatography (HPLC). Detection was carried out by tandem mass-spectrometry, monitoring two mass transitions for each enantiomer (m/z: 336 → 187 (quantifier) and 336 → 159 (qualifier)). The analysis was performed with external, matrix-matched standards.

After extraction of the honey samples from the combs, the respective specimen containers were stored at under deep-frozen conditions (≤ -18 °C).

Equipment

Instrument: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector

Column: Phenomenex Lux Cellulose-3, 3 µm, 150*2.0 mm

Column temp.: 35°C

Mobile phase: A: water containing 0.1% formic acid and 5 mM ammonia formate
B: methanol containing 0.1% formic acid and 5 mM ammonia formate

Time (min)	A %	B %
0.00	40	60
7.00	0	100
9.00	0	100
9.01	40	60
11.00	40	60

Flow rate: 0.3 mL/min

Injection volume: 10 µL

Run time: 11.00 min, 2 min re-equilibration time

Detector type: ESI positive, Multiple reaction monitoring (MRM),
Zoxamide: m/z: 336→187 (Quantifier), 336→159 (Qualifier)

Retention times: 6.8 min for R-Zoxamide; 7.6 min for S-Zoxamide

Results and discussions

Recovery findings

Summaries of the results are presented in the following table.

Table A 174: Validation results for zoxamide (racemate)

Validation level	n	Nominal conc. of ZOX [mg/kg]	Single values analysed conc. of ZOX [mg/kg]	Mean analysed conc. of ZOX [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (Quantifier)						
LOQ	5	0.010	0.0092, 0.0091, 0.0091,0.0085, 0.0082	0.0088	88	5.18
100xLOQ	5	1.002	0.923, 0.931, 0.922, 0.944, 0.920	0.928	93	1.08
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (Qualifier)						
LOQ	5	0.010	0.0091, 0.0090, 0.0091, 0.0084, 0.0086	0.0088	88	4.08
100xLOQ	5	1.002	0.945, 0.956, 0.940, 0.971, 0.946	0.951	95	1.29
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Table A 175: Validation results for R-zoxamide

Validation level	n	Nominal conc. of *R*-Zox [mg/kg]	Single values analysed conc. of *R*-Zox [mg/kg]	Mean analysed conc. of *R*-Zox [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (Quantifier)						
LOQ	5	0.005	0.0037, 0.0036, 0.0036, 0.0034, 0.0032	0.0035	70	5.43
100xLOQ	5	0.499	0.373, 0.382, 0.378, 0.389, 0.375	0.379	76	1.58
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (Qualifier)						
LOQ	5	0.010	0.0036, 0.0036, 0.0036, 0.0033, 0.0034	0.0035	70	3.34
100xLOQ	5	1.002	0.376, 0.382, 0.374, 0.387, 0.376	0.379	76	1.45
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Table A 176: Validation results for S-zoxamide

Validation level	n	Nominal conc. of *S*-Zox [mg/kg]	Single values analysed conc. of *S*-Zox [mg/kg]	Mean analysed conc. of *S*-Zox [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (Quantifier)						
LOQ	5	0.005	0.0051, 0.0050, 0.0050, 0.0045, 0.0044	0.0048	95	6.69
100xLOQ	5	0.503	0.454, 0.453, 0.448, 0.458, 0.449	0.453	90	0.88
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (Qualifier)						
LOQ	5	0.010	0.0051, 0.0050, 0.0051, 0.0044, 0.0046	0.0048	96	6.91
100xLOQ	5	1.002	0.452, 0.456, 0.450, 0.464, 0.453	0.455	90	1.23
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Accuracy and precision / repeatability

The recovery values were in the range of 70 – 110% with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

The calibration functions were linear in the range of 0.282 to 7.05 µg/L for zoxamide (racemate), in the range of 0.147 to 3.68 µg/L of R-zoxamide and in the range of 0.147 to 3.67 µg/L of S-zoxamide. 1/x weighting was applied and a correlation coefficient of > 0.99 was obtained.

Limit of quantification

The limit of quantification (LOQ) was 0.010 mg/kg of zoxamide (racemate) in honey, corresponding to 1.00 µg/L in diluted sample extracts and 0.005 mg/kg of the single enantiomers of zoxamide in honey (R-zoxamide and S-zoxamide, corresponding to 0.50 µg/L in diluted sample extracts).

Limit of detection

The limit of detection (LOD) was 0.003 mg/kg of zoxamide (racemate) and 0.0015 mg/kg of the single enantiomers of zoxamide (R-zoxamide and S-zoxamide) in honey specimens.

Matrix effects

For all analyses, matrix-matched calibration standards were used in this analytical phase.

Specificity

The method is regarded as highly specific and is able to separate the two enantiomers of zoxamide (R-zoxamide and S-zoxamide). It uses liquid chromatography (with a chiral column) with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Storage Stability of sample extracts

Stability of sample extracts was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. Sample extracts were stable for a period of 11 days at 4-10°C. The maximum storage period of sample extracts in this analytical phase was 6 days

Storage Stability of frozen samples

The maximum freezer storage period for honey samples of the field part was 83 days. The stability of honey samples in the freezer was demonstrated by recovery experiments over a storage period of at least 85 days.

Table A 177: Characteristics of the analytical method for the determination of zoxamide in honey

	Zoxamide (sum /racemate)	***R*-Zoxamide**	***S*-Zoxamide**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 22687.6 x + 8715.03, r^2=0.99986	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 313719 x + 10908.6, r^2=0.99884	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 261849 x + 39336.4, r^2=0.99884
Calibration range	0.282-7.05 µg/L in analytical samples or 0.003 to 1.41 mg/kg in honey specimens	0.147-3.68 µg/L in analytical samples or 0.0015 to 0.736 mg/kg in honey specimens	0.147-3.67 µg/L in analytical samples or 0.0015 to 0.733 mg/kg in honey specimens
Assessment of matrix effects is presented	Matrix-matched calibration standards were used. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Matrix-matched calibration standards were used. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Matrix-matched calibration standards were used. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of	LOQ: 0.010 mg/kg,	LOQ: 0.005 mg/kg,	LOQ: 0.005 mg/kg,

	Zoxamide (sum /racemate)	***R*-Zoxamide**	***S*-Zoxamide**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
determination/ quantification	corresponding to 1.00 µg/L in the analytical sample. LOD: 0.003 mg/kg, corresponding to 0.282 µg/L in the analytical sample	corresponding to 0.500 µg/L in the analytical sample. LOD: 0.0015 mg/kg, corresponding to 0.147 µg/L in the analytical sample	corresponding to 0.500 µg/L in the analytical sample. LOD: 0.0015 mg/kg, corresponding to 0.147 µg/L in the analytical sample

The following figures show typical chromatograms.

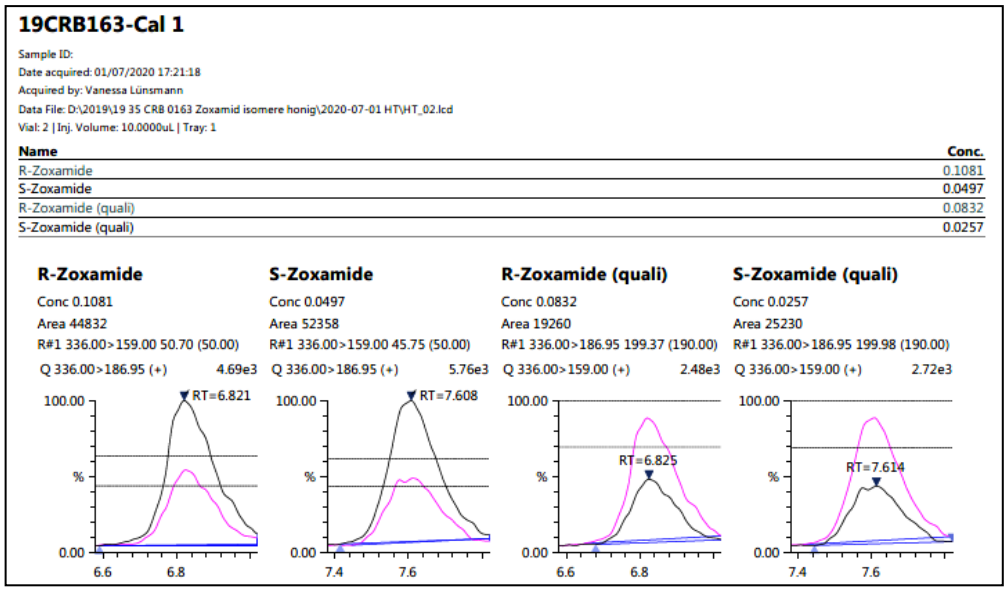


Figure A 75: Chromatogram of calibration standard Zoxamide (racemate)

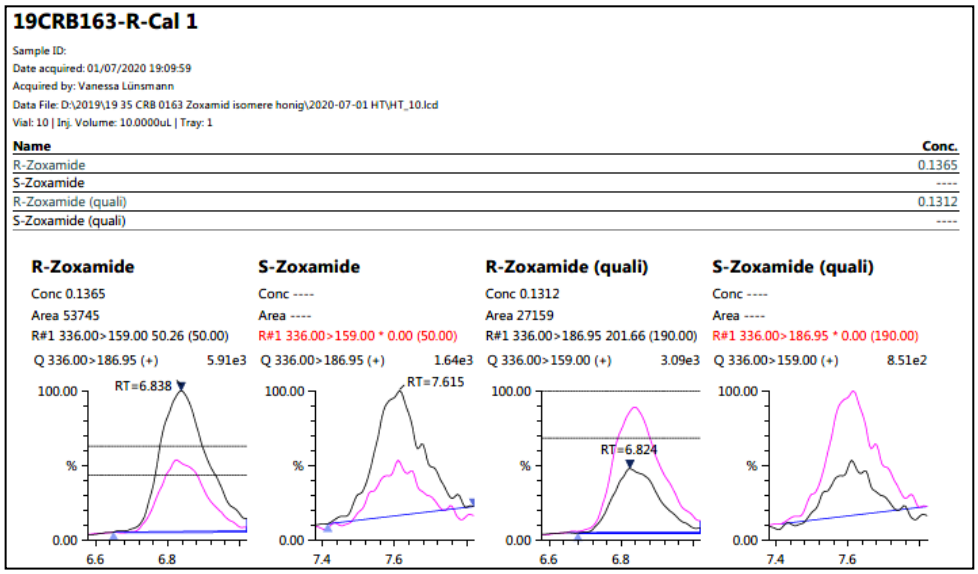


Figure A 76: Chromatogram of calibration standard R-Zoxamide

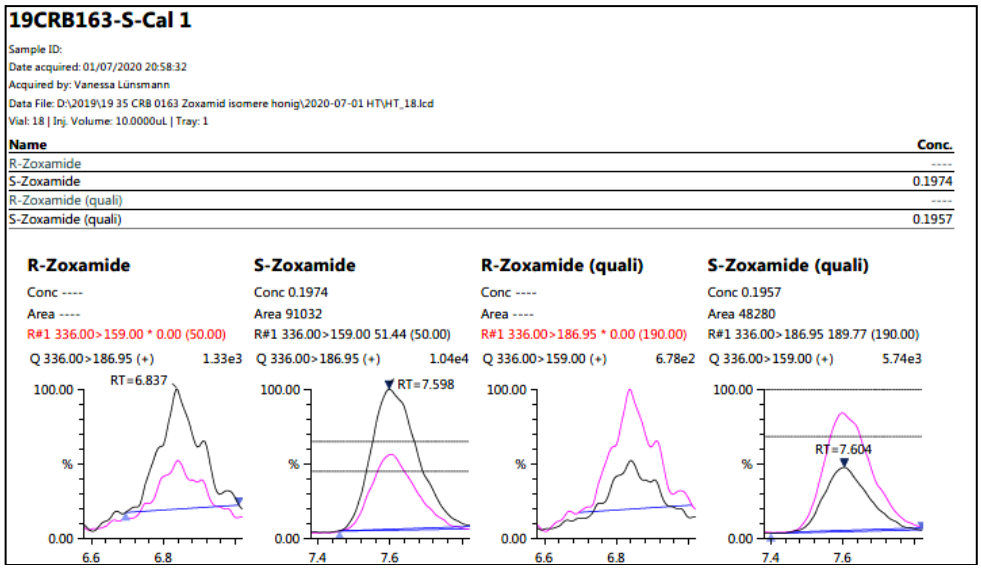


Figure A 77: Chromatogram of calibration standard S-Zoxamide

Conclusion

The method for the determination of zoxamide in honey was fully validated according to SANCO/3029/99 rev. 4 (2000) and SANCO/825/00 rev. 8.1 (2010) with a limit of quantification (LOQ) of 0.01 mg/kg and a limit of detection (LOD) of 0.003 mg/kg.

(Poráczki K. 2020)

A 2.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new data.

A 2.1.2.3 Description of methods for the analysis of soil (KCP 5.2)

No new data.

A 2.1.2.4 Description of methods for the analysis of water (KCP 5.2)

No new data.

A 2.1.2.5 Description of methods for the analysis of air (KCP 5.2)

No new data.

A 2.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

EFSA (2017) has requested *"An analytical method for monitoring zoxamide in body fluids and tissues (relevant for all representative uses evaluated; submission date proposed by the applicant: unknown; see Section 1)."* This method is provided.

A 2.1.2.6.1 Analytical method 1 – determination of zoxamide in body fluids and tissues

A 2.1.2.6.1.1 Method validation

Comments of zRMS:	Confirmatory study for zoxamide currently being evaluated by RMS (Latvia).

Reference:	**KCP 5.2/04**
Report	Coleman, H., 2017: Validation of an analytical method for the determination of zoxamide in body fluid and tissue Gowan Crop Protection Ltd., Berkshire, UK Battelle UK Ltd., Report No. FF/17/002, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010)
Deviations:	None
Acceptability:	Yes

Materials and methods

The objective of this study was to validate a method for the determination of residues of zoxamide in body fluids (urine) and tissues (bovine liver) according to SANCO 825/00 rev. 8.1 (2010).

For the analysis of urine, aliquots of 2.0 mL were placed into glass vials. 2 mL acetonitrile was added to each vial and the sample mixed using a vortex mixer. 1 mL of the sample was transferred to a separate glass vial and 4 mL acetonitrile/water (1:1, v/v) was added. After vortex mixing, an aliquot was transferred to an HPLC vial. Final residue levels were determined by LC-MS/MS with an LOQ of 0.05 mg/L using matrix-matched standards.

Residues of zoxamide in tissue were determined using the QuEChERS extraction procedure with subsequent d-SPE clean up. Aliquots of 5 g of tissue were weighed into 50 mL centrifuge tubes. 10 mL water and 10 mL acetonitrile were added, and the mixture was shaken vigorously by hand for 1 minute. Each sample was shaken vigorously by hand for 1 minute with a packet of QuEChERS citrate salt mixture (4.0 g magnesium sulphate, 1.0 g sodium chloride, 1.0 g sodium citrate tribasic dihydrate and 0.5 g sodium citrate dibasic sesquihydrate). After centrifugation for 3 minutes at 4000 rpm, 1.5 mL of the supernatant was added to a d-SPE QuEChERS tube containing 150 mg magnesium sulphate, 25 mg primary and secondary amine exchange material (PSA) and 25 mg C18E. The sample was then vortex mixed for 30 seconds before centrifugation for 2 minutes at 4000 rpm. A 1 mL aliquot from the d-SPE tube was mixed with 9 mL acetonitrile: water (1:1; v/v) by a vortex mixer. An aliquot was transferred to an HPLC vial and final residue levels were determined by LC-MS/MS with a LOQ of 0.1 mg/kg against matrix-matched standards.

Typical liquid chromatography conditions with tandem mass spectrometry (LC-MS/MS) and monitoring data for two ion mass transitions - for quantification and/or confirmation - are summarised in the following.

Equipment

LC-MS/MS:	API 6500 Triple Quadrupole Mass Spectrometer with Turbo spray ion source
Column:	Restek Ultra Aqueous C18, 100 x 2.1 mm, 3 µm

Mobile phase: A: 10 mM Ammonium acetate in water
B: 10 mM Ammonium acetate in methanol

Time (min)	% A	% B
0.0	80	20
1.0	10	90
4.5	0	100
4.8	0	100
4.9	80	20

Flow rate: 0.5 mL/min
Column temp.: 35°C
Injection volume: 10 µL
Retention time: Approximately 2.2-2.4 minutes
Ion mode: Zoxamide:
Positive mode
m/z 336/187
m/z 336/124 (urine); m/z 336/159 (liver)

For detailed mass spectrometer conditions, please refer to the study report.

Results and discussions

Accuracy and precision

Recovery findings

Results of the recovery experiments are presented in the following table.

Table A 178: Recovery results for zoxamide

Matrix	Transition	Nominal Fortification Level	Mean Recovery Efficiency (%)	SD (%)	RSD (%)	n
Human Urine	336/187	0.05 mg/L	79.3	1.9	2.4	6
	336/124	0.05 mg/L	79.0	2.8	3.6	6
Tissue (Bovine liver)	336/187	0.1 mg/kg	101	1.9	1.9	6
	336/159	0.1 mg/kg	101	1.5	1.5	6

SD = standard deviation; RSD = relative standard deviation; n = sample size

The results obtained at the LOQ levels were in an acceptable range of 70 – 110 % with relative standard deviations (RSDs) ≤ 20%, which demonstrate acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for zoxamide for both mass transitions and commodities. The calibration ranges were between 1.50 – 6.00 ng/mL with 7 concentration levels for each matrix-matched calibration curve. This covers ranges from at least 30 % of the LOQ to at least 20 % above the highest concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearities.

Specificity

The method is regarded as specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as zoxamide, were

either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ). Example LC-MS/MS chromatograms of external standard, control (untreated urine and tissue) and fortified matrix solutions are presented in the report.

Limit of quantification

The limit of quantification (LOQ) was established and validated at 0.05 mg/L for urine and 0.1 mg/kg for tissue (bovine liver) extracts.

Limit of detection

The limit of detection (LOD) for each matrix was defined as the lowest quantifiable calibration standard. It was 0.02 mg/L for urine and 0.03 mg/kg for tissue (bovine liver).

Matrix effects

Matrix effects were investigated at the LOQ, by comparing peak areas of a solvent standard solution to peak areas of matrix-matched standard solutions for each matrix at equivalent concentrations. They were below 20% suppression/enhancement and therefore regarded insignificant for both transitions. Thus, solvent standard calibration lines may be used for quantification of zoxamide. However, matrix standards were used to make the method more robust.

Storage stability of sample extracts and solvent standards

Zoxamide in urine extracts was stable for at least 11 days when stored at nominal temperatures of 4 °C (in the refrigerator), and zoxamide in tissue extracts for at least 22 days. Stock solutions of zoxamide in acetone were shown to be stable for at least 15 days when stored at nominal temperatures of 4 °C (in the refrigerator).

Table A 179: Characteristics of the method validation for the analysis of zoxamide in urine and (liver) tissues

	Zoxamide
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Standard solution and matrix matched calibration. Calibration range 1.50 – 6.00 ng/mL linear (7 points) Correlation coefficient $r^2 > 0.99$ for both (primary and/or confirmatory) transitions. Individual calibration data and calibration line equations presented in the study report.
Calibration range	1.50 - 6.00 ng/mL
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.05 mg/L for urine and 0.1 mg/kg for (liver) tissue LOD: 0.02 mg/L for urine and 0.03 mg/kg for (liver) tissue

The following figure shows a representative chromatogram.

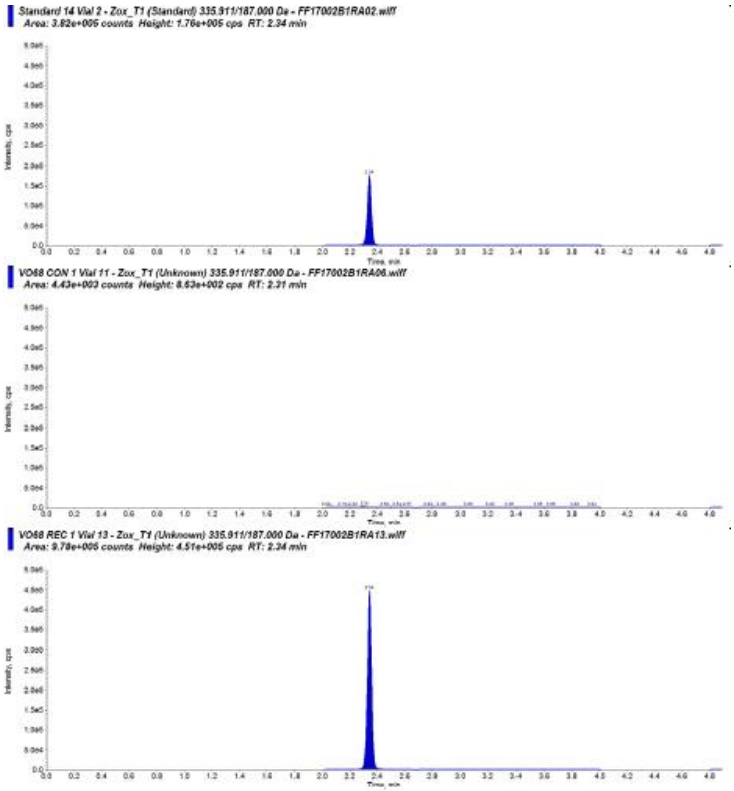


Figure A 78: Representative chromatogram

Conclusion

A method for the determination of residues of zoxamide in body fluids (urine) and tissues (bovine liver) was successfully validated according to SANCO 825/00 rev. 8.1 (2010) in terms of linearity, specificity, LOQ, accuracy, precision, matrix effects and stability of extract and standard sample solutions. Satisfactory validation data were generated for two ion mass transitions, demonstrating that either may be used for quantification and/or confirmation.

As a result, the method is considered suitable for the determination of residues of zoxamide in (human) urine and (bovine liver) tissue with an LOQ of 0.05 mg/L and 0.1 mg/kg, respectively.

(Coleman H. 2017)

A 2.1.2.7 Other Studies/ Information

No new data.

A 2.2 Analytical methods for cymoxanil

A 2.2.1 Methods used for the generation of pre-authorisation data (KCP 5.1)

A 2.2.1.1 Description of analytical methods for the determination of residues in water, aqueous buffer solutions, aquatic media

A 2.2.1.1.1 Analytical method 1

A 2.2.1.1.1.1 Method validation

Comments of zRMS:	Study 5.1/45 was already provided and assessed during the product authorisation

Reference:	**KCP 5.1/45**
Report	xxx, 2007: Acute toxicity of Cymoxanil 33% + Zoxamide 33% WG to rainbow trout (*Oncorhynchus mykiss*), determined under flow-through conditions. Oxon Italia S.p.A., Italy xxx, Report No. CH-E-023/2006, GLP, Not published
Guideline(s):	OECD 203 (1992) EU Commission Directive 92/69/EEC, C1 (1992) OPPTS 850.1075 (1996)
Deviations:	No
GLP:	Yes
Acceptability:	Yes
Duplication (if vertebrate study)	No

Materials and methods

ChemService S.r.I. conducted a study to determine the test article content in water samples coming from the ecotoxicological tests performed by ChemService Ecotox Department.

The study was performed in accordance with analytical phase Study Protocol CH-157/2006 and was fully validated according to SANCO guidance's 3029/99 rev. 4 and 825/00 rev. 7.

In a preliminary non GLP phase (study No. CH-156/2006), it was found that injecting standard and water extract samples, two major peaks were obtained in GC/MS analysis, each of them attributed to zoxamide because of the presence of characteristic ions. After several tests with different injector temperatures and taking into consideration that the high grade certified analytical standard eluted in these two peaks, it was finally supposed that there a probable degradation of the active ingredient in the heated liner into the split-splitless injector.

To avoid or at least to limit this degradation without a contemporary loss of sensitivity, as best compromise condition was chosen to take into consideration the contribution of both peaks by a sum of the peak areas of the common ion at 187 m/z using the selected ions monitoring (SIM) technique.

The samples were stored at 4°C for 30 days.

Equipment

Instrument:	Gas chromatograph Thermo Finnigan Mod. Trace GCUltra, equipped with split/splitless injector, autosampler AS3000, and coupled with a Thermo Finnigan Polaris Q ion trap mass detector

For further MS conditions, please refer to the study report.

Results and discussions

Table A 180: Summary results

ChemService No.	Time	Type (*)	Zoxamide (µg/L)	Formulation (µg/L)	Recovery vs. nominal
AA29019	0 h	Control	n.d.	-	-
AA29020	0 h	Test concentration 0.10 mg/L	16.02	48.55	48.5%
AA29021	0 h	Test concentration 0.32 mg/L	71.10	215.45	67.3%
AA29022	0 h	Test concentration 1.00 mg/L	106.87	323.85	32.4%
AA29023	0 h	Test concentration 3.20 mg/L	188.02	569.76	17.8%
AA29024	0 h	Test concentration 10.00 mg/L	294.23	891.61	8.9%
AA29025	48 h	Control	n.d.	-	-
AA29026	48 h	Test concentration 0.10 mg/L	29.20	88.48	88.5%
AA29027	48 h	Test concentration 0.32 mg/L	69.58	210.85	65.9%
AA29028	48 h	Test concentration 1.00 mg/L	118.92	360.36	36.0%
AA29029	48 h	Test concentration 3.20 mg/L	163.36	495.03	15.5%
AA29030	48 h	Test concentration 10.00 mg/L	237.39	719.36	7.2%
AA29031	72 h	Test concentration 10.00 mg/L	209.94	636.18	6.4%
AA29032	96 h	Control	n.d.	-	-
AA29033	96 h	Test concentration 0.10 mg/L	19.53	59.18	59.2%
AA29034	96 h	Test concentration 0.32 mg/L	68.06	206.24	64.5%
AA29035	96 h	Test concentration 1.00 mg/L	124.98	378.73	37.9%
AA29036	96 h	Test concentration 3.20 mg/L	155.11	470.03	14.7%

* The nominal concentration for ecotoxicological fortification samples is expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.

Accuracy and precision / repeatability

For accuracy, the SANCO guideline requires mean recovery values in the range from 70 to 110% at each level; the slight deviation obtained can be accepted because of the low water solubility of the test substance; therefore, the accuracy of the analytical method was considered to be acceptable.

For the precision, the SANCO guideline requires an RSD % lower than 20 % for each level.

Linearity

The range tested was from 20 to 400 ng/ml, corresponding to zoxamide concentrations from 10 to 200 µg/L in the water samples, was found to be linear (correlation coefficient > 0.99).

Limit of quantification

The limit of quantification (LOQ) of this method is defined as the lowest fortification level for which recovery in the range of 70-110 % and CC < 20 % are obtained, was found to be of 10 µg/L in water matrix samples, corresponding to a final solution of 20 ng/ml (ppb).

Limit of detection

The limit of detection (LOD) of this method is defined as 50% of the lowest calibration level, i.e. 10 ng/ml (ppb) corresponding to 5 µg/L in the water matrix samples.

Matrix effects

Matrix-matched standards were used.

Specificity

The GC MS method is regarded specific.

Storage stability

The samples were stored at 4°C for 30 days.

Table A 181: Characteristics of the analytical method validations for zoxamide in aquatic media

	Zoxamide
Specificity	The GC MS method is regarded specific Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	5-point calibration with external matrix-matched standard The calibration was linear. $r^2 = 0.99990$
Calibration range	10 to 200 µg/L
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 10 µg/L LOD: 5 µg/L

The following figures show typical chromatograms.

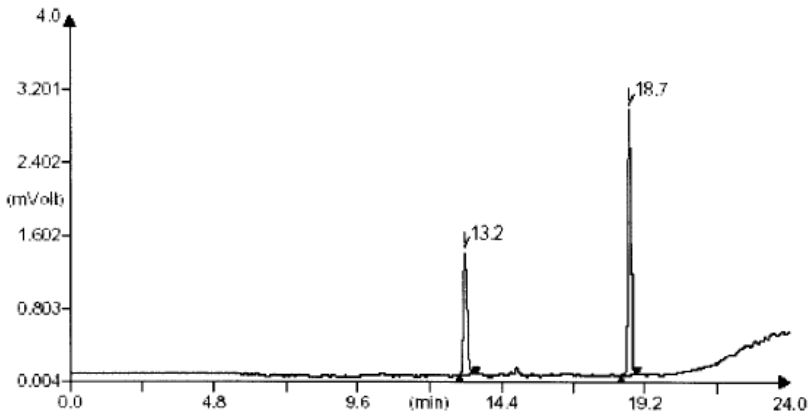


Figure A 79: **Representative chromatogram of the standard solution of zoxamide**

Conclusion

The analytical method was shown to be specific for zoxamide residues in water samples from the aquatic ecotoxicological studies.

(xxx. 2007)

A 2.2.1.1.2 Analytical method 2

A 2.2.1.1.2.1 Method validation

Comments of zRMS:	The studies 5.1/46 and 47 were already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/46**
Report	Croce, V., 2007: Acute toxicity of Cymoxanil 33% + Zoxamide 33% WG to *Daphnia magna* in a 48-hour immobilization test under semi-static exposure - limit test Oxon Italia S.p.A., Italy ChemService S.r.I., Italy, Report No. CH-E-001/2007, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000) SANCO 825/00 rev. 7
Deviations:	No
Acceptability:	Yes

and

Reference:	**KCP 5.1/47**
Report	Croce, V., 2007: Toxicity of Cymoxanil 33% + Zoxamide 33% WG to green algae *Pseudokirchneriella subcapitata* determined in a growth inhibition study Oxon Italia S.p.A., Italy ChemService S.r.I., Italy, Report No. CH-E-002/2007, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000) SANCO 825/00 rev. 7
Deviations:	No
Acceptability:	Yes

Materials and methods

ChemService S.r.I. conducted a study to determine the test article content in water samples coming from the ecotoxicological tests performed by ChemService Ecotox Department.

All controls and fortified water samples were extracted and analysed following ChemService Analytical Method No. 156/2006. The method was fully validated according to SANCO 3029/99 rev. 4 and SANCO 825/00 rev. 7

In a preliminary non GLP phase (study No. CH-156/2006), it was found that injecting standard and water extract samples, two major peaks were obtained in GC/MS analysis, each of them attributed to zoxamide because of the presence of characteristic ions. After several tests with different injector temperatures and taking into consideration that the high grade certified analytical standard eluted in these two peaks, it was finally supposed that there a probable degradation of the active ingredient in the heat-ed liner into the split-splitless injector.

To avoid or at least to limit this degradation without a contemporary loss of sensitivity, as best com-promise condition was chosen to take into consideration the contribution of both peaks by a sum of the peak areas of the common ion at 187 m/z using the selected ions monitoring (SIM) technique.

The samples were stored at 4°C for 30 days.

Equipment

Instrument: Gas chromatograph Thermo Finnigan Mod. Trace GCUltra, equipped with split/splitless injector, autosampler AS3000, and coupled with a Thermo Finnigan Polaris Q ion trap mass detector

Results and discussions

Table A 182: Summary results in ecotoxicological water samples (daphnia magna)

ChemService No.	Time	Type (*)	Zoxamide (mg/L)	Formulation. (mg/L)	Recovery vs. nominal
AA35186	0 h	Control (time 0)	n.d.	-	-
AA35187	0 h	Test concentration 100 mg/L (time O)	22.69	68.7	69 %
AA35188	24 h old	Control (after 24h)	n.d.	-	-
AA35189	24 h old	Test concentration 100 mg/L (after 24h)	8.39	25.4	25 %
AA35190	24 h new	Control (new solution after 24 h)	n.d.	-	-
AA35191	24 h new	Test concentration 100 mg/L (new solution after 24 h)	22.97	69.6	70 %
AA35192	48 h	Control (after 48h)	n.d.	-	-
AA35193	48 h	Test concentration 100 mg/L (after 48h)	10.68	32.4	32 %

(*) The nominal concentration for ecotoxicological fortification samples are expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.
n.d. not detected, lower than LOD (5 µg/L)

Table A 183: Summary results in ecotoxicological water samples (green algae)

ChemService No.	Time	Type (*)	Zoxamide (mg/L)	Formulation (mg/L)	Recovery vs. nominal
AA35195	0 h	Control t0	n.d.	-	-
AA35196	0 h	Test concentration 0.03 mg/L	0.021	0.062	210
AA35197	0 h	Test concentration 0.07 mg/L	0.051	0.156	255
AA35198	0 h	Test concentration 0.17 mg/L	0.071	0.216	118
AA35199	0 h	Test concentration 0.41 mg/L	0.132	0.399	94
AA35200	0 h	Test concentration 1.00 mg/L	0.331	1.002	100
AA35201	72 h	Control t72h	n.d.	-	-
AA35202	72 h	Test concentration 0.03 mg/L	0.008	0.025	80
AA35203	72 h	Test concentration 0.07 mg/L	0.036	0.109	165
AA35204	72 h	Test concentration 0.17 mg/L	0.039	0.118	65
AA35205	72 h	Test concentration 0.41 mg/L	0.908	0.275	65
AA35206	72 h	Test concentration 1.00 mg/L	0.168	0.509	51
AA35207	72 h	Test concentration 0.03 mg/L WAI	0.042	0.127	170
AA35208	72 h	Test concentration 0.07 mg/L WAI	0.031	0.095	195
AA35209	72 h	Test concentration 0.17 mg/L WAI	0.057	0.171	95
AA35210	72 h	Test concentration 0.41 mg/L WAI	0.117	0.354	84
AA35211	72 h	Test concentration 1.00 mg/L WAI	0.216	0.654	65

(*) The nominal concentration for ecotoxicological fortification samples are expressed as formulation Cymoxanil 33% + Zoxamide 33% WG.
n.d. not detected, lower than LOD (5 µg/L)
WAI: without algal inoculum.

Accuracy and precision / repeatability

For accuracy, the SANCO guideline requires mean recovery values in the range from 70 to 110% at each level; the slight deviation obtained can be accepted because of the low water solubility of the test substance; therefore, the accuracy of the analytical method was considered to be acceptable.

For the precision, the SANCO guideline requires an RSD % lower than 20 % for each level.

Linearity

The range tested was from 20 to 400 ng/ml, corresponding to zoxamide concentrations from 10 to 200 µg/L in the water samples, was found to be linear (correlation coefficient > 0.99).

Limit of quantification

The limit of quantification (LOQ) of this method is defined as the lowest fortification level for which recovery in the range of 70-110 % and CC < 20 % are obtained, was found to be of 10 µg/L in water matrix samples, corresponding to a final solution of 20 ng/ml (ppb).

Limit of detection

The limit of detection (LOD) of this method is defined as 50% of the lowest calibration level, i.e. 10 ng/ml (ppb) corresponding to 5 µg/L in the water matrix samples.

Matrix effects

Matrix-matched standards were used.

Specificity

The GC MS method is regarded specific.

Storage stability

The samples were stored at 4°C for 30 days.

Table A 184: Characteristics of the analytical method validations for zoxamide in aquatic media

	Zoxamide
Specificity	The GC MS method is regarded specific Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	5-point calibration with external matrix-matched standard The calibration was linear. r^2 = 0.99990
Calibration range	10 to 200 µg/L
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 10 µg/L LOD: 5 µg/L

The following figures show typical chromatograms.

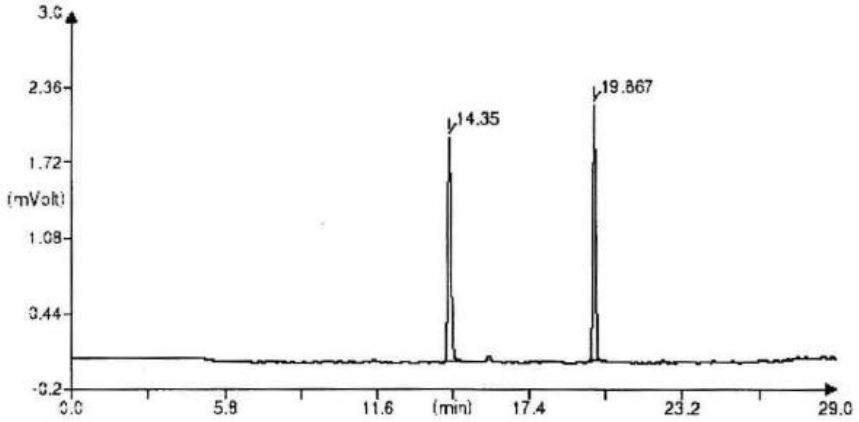


Figure A 80: **Representative chromatogram of the standard solution of zoxamide**

Conclusion

The analytical method was shown to be specific for zoxamide residues in water samples from the aquatic ecotoxicological studies.

(xxx 2007)

A 2.2.1.1.3 Analytical method 3

A 2.2.1.1.3.1 Method validation

Comments of zRMS:	The study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/50**
Report	xxx, 2007: Cymoxanil 33% + Zoxamide 33% WG: validation of the analytical method for the determination of the content of zoxamide in water samples from the aquatic ecotoxicological studies Oxon Italia S.p.A., Italy xxx., Report No. CH-156/2006, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANCO/825/00 rev. 7 (2004)
Deviations:	None
Acceptability:	Yes

Materials and methods

ChemService S.r.I. conducted a study to adjust and internally re-validate the analytical method DAS 34-98-52 (RCC project No. 692256) for the determination of the content of the active substance, zoxamide, in water samples from the aquatic ecotoxicological studies performed in accordance with the guidelines SANCO/3029/99 rev. 4 (2000) and SANCO/825/00 rev. 7 (2004).

An aliquot of 10 ml of the water sample was passed through a suitably conditioned 200 mg Lichrolut EN solid phase extraction (SPE) cartridge. The cartridge was dried and then eluted with 10 ml of ethyl acetate. The organic extract was concentrated at 5 mL and injected into a gas chromatograph coupled with a Mass Detector (MS).

The reference material was stored at -20°C into the freezer.

Equipment:

LC-MS/MS:	API 6500 Triple Quadrupole Mass Spectrometer with Turbo spray ion source
Column:	ChemServiee code No. 139
Flow rate:	1 mL/min
Column temp.:	5°C/min from 150°C to 250°C 20°C/min from 250°C to 300°C 2 min at 300°C
Injection volume:	1 µL
Total analysis time:	24 min
Detector:	Polaris Q MS, SIM mode (187 m/z)

For detailed mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 185: Recovery at low fortification level (10.56 µg/L zoxamide a.s.)

Code Number	As	C_s (1) (ng/mL)	V_s (mL)	V_w (mL)	D_s (mL)	Zoxamide (µg/L)	Recovery (%) *
Control	0	-	5	10	1	n.d.	-
Spike L A	34427	19.65	5.0	10	1	9.823	93.02
Spike L B	33500	19.12	5.0	10	1	9.559	90.52
Spike L C	35350	20.17	5.0	10	1	10.087	95.52
Spike L D	29792	17.00	5.0	10	1	8.501	80.50
Spike L E	32696	18.66	5.0	10	1	9.330	88.35
Spike L F	33953	19.38	5.0	10	1	9.688	91.74
Spike L G	31152	17.78	5.0	10.0	1	8.889	84.18
Spike L H	29003	16.55	5.0	10.0	1	8.276	78.37
					Mean value:	**9.27**	**87.8**
					Standard deviation (SD):	**0.61**	**5.76**
					Coefficient of Variation (CV %):	**6.6%**	**6.6%**

• corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples and with the lowest standard calibration level for control samples.
n.d. not detected, lower than LOD (5 µg/L)

Table A 186: Recovery at low fortification level (105.7 µg/L zoxamide a.s.)

Code Number	As	Cs (1) (ng/mL)	Vs (mL)	V_w (mL)	Ds (mL)	Zoxamide (µg/L)	Recovery (%) *
Control	0	-	10	10	1	n.d.	-
Spike M A	151531	86.45	10.0	10.0	1	86.454	81.79
Spike M B	169523	95.62	10.0	10.0	1	95.623	90.47
Spike M C	159146	90.33	10.0	10.0	1	90.335	85.46
Spike M D	182998	102.49	10.0	10.0	1	102.490	96.96
Spike M E	166152	93.91	10.0	10.0	1	93.905	88.84
Spike M F	183261	102.62	10.0	10.0	1	102.624	97.09
Spike M G	196745	109.50	10.0	10.0	1	109.496	103.59

Spike M H	186124	104.08	10.0	10.0	1	104.083	98.47
					Mean value:	**98.13**	**92.8**
					Standard deviation (SD):	**7.29**	**6.90**
					Coefficient of Variation (CV %):	**7.4%**	**7.4%**

• corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples and with the lowest standard calibration level for control samples.
n.d. not detected, lower than LOD (5 µg/L)

Table A 187: Recovery at low fortification level (317 µg/L zoxamide a.s.)

Code Number	As	Cs (1) (ng/mL)	Vs (mL)	V_w (mL)	Ds (mL)	Zoxamide (µg/L)	Recovery (%) *
Control	0	-	10	10	1	n.d.	-
Spike H A	571312	305.43	10.0	10.0	1	305.426	96.35
Spike H B	541222	289.65	10.0	10.0	1	289.651	91.37
Spike H C	593858	317.25	10.0	10.0	1	317.246	100.08
Spike H D	596984	318.89	10.0	10.0	1	318.885	100.59
Spike H E	549029	293.74	10.0	10.0	1	293.744	92.66
Spike H F	543945	291.08	10.0	10.0	1	291 .079	91.82
Spike H G	567696	303.53	10.0	10.0	1	303.530	95.75
Spike H H	533466	285.58	10.0	10.0	1	285.585	90.09
					Mean value:	300.64	94.8
					Standard deviation (SD):	11.87	3.74
					Coefficient of Variation (CV%):	3.9%	3.9%

• corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples and with the lowest standard calibration level for control samples.
n.d. not detected, lower than LOD (5 µg/L)

Table A 188: Recovery at low fortification level (357.72 µg/L zoxamide a.s.)

Code Number	As	Cs (1) (ng/mL)	Vs (mL)	V_w (mL)	Ds (mL)	Zoxamide (µg/L)	Recovery (%) *
Control	0	-	100.0		1000	n.d.	-
Solution.1	691165	367.26	100.0	108.4	1000	367.26	102.7%
Solution.2	684976	364.01	100.0	108.4	1000	364.01	101.8%
Solution.3	648054	344.64	100.0	108.4	1000	344.64	96.3%
Solution.4	680884	361.86	100.0	108.4	1000	361.86	101.2%
Solution.5	635141	337.86	100.0	108.4	1000	337.86	94.4%
Solution.6	705918	375.00	100.0	108.4	1000	375.00	104.8%
Solution.7	661712	351.80	100.0	108.4	1000	351.80	98.3%
Solution.8	665631	353.86	100.0	108.4	1000	353.86	98.9%
					Mean value:	**357.04**	**99.8%**
					Standard deviation (SD):	**11.50**	**0.0321**
					Coefficient of Variation (CV %):	**3.2%**	**3.2%**

• corrected for mean control residue value
(1) Quantification with the linear calibration curve for fortified samples and with the lowest standard calibration level for control samples.
n.d. not detected, lower than LOD (5 µg/L)

Accuracy and precision

Both repeatability and recovery tests were performed using 10 ml control samples of the re-constituted

water freshly fortified at three zoxamide nominal levels, 10 µg/L, 100 µg/L and 300 µg/L, within the linearity test.

The accuracy of the analytical method was considered to be acceptable being mean recovery for all fortification in the range of 88-100 %.

Linearity

The range tested was from 20 to 400 ng/ml was found to be linear (correlation coefficient > 0.99); this range is suitable to analyse zoxamide in ecotox medium at level from 10 µg/L to 330 mg/L, after dilution where necessary.

Limit of quantification

The limit of quantification (LOQ) of this method is defined as the lowest fortification level for which recovery in the range of 70-110 % and CC < 20 % are obtained, was found to be of 10 µg/L in water matrix samples, corresponding to a final solution of 20 ng/ml (ppb).

Limit of detection

The limit of detection (LOD) of this method is defined as 50% of the lowest calibration level, i.e. 10 ng/mL (ppb) corresponding to 5 µg/L in the water matrix samples.

Matrix effects

Matrix effects were investigated at the LOQ, by comparing peak areas of a solvent standard solution to peak areas of matrix-matched standard solutions for each matrix at equivalent concentrations. They were below 20% suppression/enhancement and therefore regarded insignificant for both transitions. Thus, solvent standard calibration lines may be used for quantification of zoxamide. However, matrix standards were used to make the method more robust.

Specificity

A comparison of the chromatograms of the zoxamide analytical standard, solvent wash, control water sample and water sample spiked at 300 µg/L, did not show any interference. Therefore, by using the conditions stated in the method, interferences can be avoided and the zoxamide residue can be determined reliably in water samples

Table A 189: Characteristics of the method validation for the analysis of zoxamide in water

	Zoxamide
Specificity	LC.MS/MS method regarded highly specific. Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Matrix matched standard calibration. 5 points calibration; r^2>0.99; linear Individual calibration data and calibration line equation presented in the study report
Calibration range	20 to 400 ng/ml (10 µg/L to 330 mg/L)
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 10 µg/L LOD: 5 µg/L

The following figure shows a representative chromatogram.

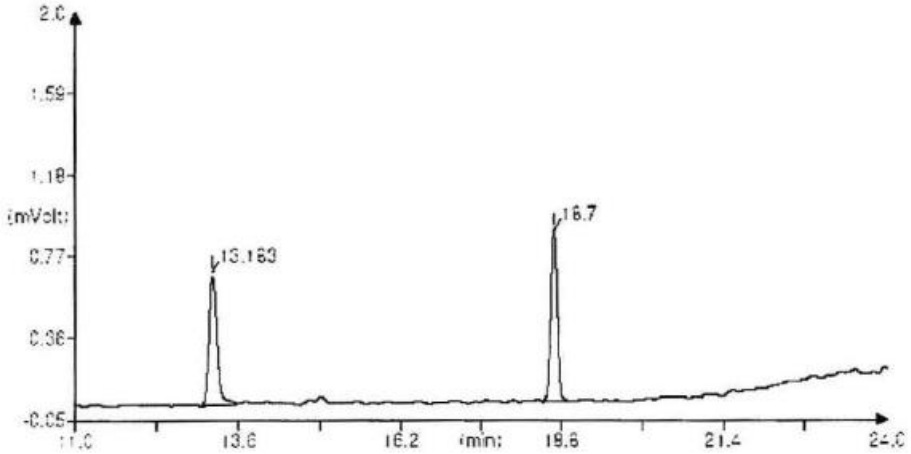


Figure A 81: Representative chromatogram

Conclusion

The analytical method was shown to be specific for zoxamide residues in water samples from the aquatic ecotoxicological studies performed, in accordance with the requirements of the Council Directive 91/414/EEC, by the Ecotoxicological Studies Department of ChemService.

In conclusion the GC/MS method used to analyse zoxamide in ecotox medium was successfully validated for the concentrations ranging from 10 µg/L to 330 mg/L.

(xxx 2007)

A 2.2.1.2 Description of analytical methods for the determination of residues in soil

A 2.2.1.2.1 Analytical method 1

A 2.2.1.2.1.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/56**
Report	Thomas, H., 2020: Validation of a HPLC-MS-MS method for the determination of cymoxanil and zoxamide in soil Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A. BioChem agrar, Germany, Report No.18 35 CRX 0033, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The objective of the study was the development and validation of an analytical method for the determination of cymoxanil and zoxamide, the active ingredients in Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823; trade name e.g. REBOOT), to confirm the applied test item concentrations in soil specimens of ecotoxicological studies.

The method was developed based on Jooß (2013) and Melkebeke (2000), using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC), and tandem mass (MS/MS) determination of the analytes with matrix-matched external standards. It is regarded as highly specific for the determination of the analytes with two mass transitions (cymoxanil: m/z 199.1 → 128.1 and 199.1 → 111.0, zoxamide: m/z 336.0 → 187.0 and 336.0 → 159.0), for quantification and/or qualification, respectively. The limit of quantification (LOQ) was chosen at 0.0125 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. The method was fully validated at the LOQ (0.0125 mg/kg soil d.w.), 10x LOQ (0.125 mg/kg soil d.w.) and 100x LOQ (1.25 mg/kg soil d.w.) according to guidance document SANCO/3029/99 rev. 4 (11/07/2000) using two different soil types, a sandy loamy silt from Saxony and an artificial soil substrate with 10 % peat.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugated. Aliquots of the acetonitrile phase were diluted with a solution of 50:50 (v/v) methanol/water containing 0.1% formic acid to the validated concentration range and analysed.

An external calibration (2.0 to 30 µg/L for both cymoxanil and zoxamide) with the reference items was performed. The calibration spanned from less than 80% of the lowest validation concentration to more than 120% of the highest validation concentration (taking into account the sample preparation and dilution).

Equipment

Instrument:	Triple Qudrupole Shimadzu LCMS-8040
Column:	ACE Excel 3 C18-AR, 100 * 2.1 mm
Mobile phase:	A: water containing 0.1 % formic acid and 5 mmol/L ammonium formate B: methanol containing 0.1 % formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate:	0.4 mL/min
Column temp.:	40°C
Injection volume:	1 µL
Retention time:	Cymoxanil: approx. 3.95 min Zoxamide: approx. 6.05 min
Run time:	9.0 min
Ionisation:	ESI (electrospray ionisation) positive
Ion mode	Cymoxanil: m/z 199.1 → m/z 128.1 (quantifier ion) m/z 199.1 → m/z 111.0 (qualifier ion) Zoxamide:

m/z 336 → m/z 187 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 190: Recovery results from method validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg a.i. in moist soil)	n	Mean recovery (%)	RSD (%)	Comments
Soil	Cymoxanil	0.01	2 x 5	91	9.5	Pooled RSD from two soils
Soil	Cymoxanil	0.10	2 x 5	94	2.0	Pooled RSD from two soils
Soil	Cymoxanil	1.01	2 x 5	92	1.3	Pooled RSD from two soils
Soil	Zoxamide	0.01	2 x 5	98	8.6	Pooled RSD from two soils
Soil	Zoxamide	0.10	2 x 5	96	0.7	Pooled RSD from two soils
Soil	Zoxamide	1.01	2 x 5	91	0.9	Pooled RSD from two soils

RSD = relative standard deviation
n = sample size

Accuracy and precision / repeatability

The results were in the range of 70 – 110 % with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for both analytes over the whole calibration range. The calibration ranges were between 2.0 to 30 µg/L with 6 concentration levels for each matrix-matched calibration curve (corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight).This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r^2) all greater than 0.999, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 0.01 mg/kg a.i. in moist soil, equivalent to 0.0125 mg/kg dry weight.

Limit of detection

The limit of detection (LOD) is estimated at 0.003 mg/kg a.i. for cymoxanil and zoxamide (0.004 mg/kg dry weight).

Matrix effects

Matrix effects were evaluated during non-GLP method development. Standards in soil extract matrix showed a reduction in peak area of about 27% for cymoxanil and about 10% for zoxamide compared to standards in solvent. Matrix-matched standard solutions were therefore used for the quantification of cymoxanil and zoxamide in the sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as zoxamide or cymoxanil were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for at max. 8 days in the cooled autosampler. Therefore, recovery experiments were performed and demonstrated that the analytes in soil extracts were stable for at least 8 days when stored at temperatures of 4-8 °C (in the refrigerator or the cooled autosampler).

Storage stability of frozen samples

The stability of frozen samples is investigated in a separate study.

Table A 191: Characteristics of the analytical method validation for the determination of zoxamide and cymoxanil in artificial soil

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point calibration with external matrix-matched standard The calibration was linear. Calibration curve equation: $y = 1602.523 x + 45.27193$, $r^2 = 0.99990$	6-point calibration with external matrix-matched standard The calibrations was linear. Calibration curve equation: $y = 63612.26 x + 27042.29$, $r^2 = 0.99997$
Calibration range	2.0 to 30 µg/L in soil extracts, corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight	2.0 to 30 µg/L in soil extracts, corresponding to 0.008 – 0.120 mg/kg in moist soil or 0.01 – 0.15 mg/kg dry weight
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had no peaks >30% of the lowest validation samples.	Recoveries of validation samples within 70-110%. Validation blank samples had no peaks >30% of the lowest validation samples.
Limit of determination/ quantification	LOQ: 0.01 mg/kg moist soil; 0.0125 mg/kg soil dry weight LOD: 0.003 mg/kg moist soil, 0.004 mg/kg soil dry weight	LOQ: 0.01 mg/kg moist soil; 0.0125 mg/kg soil dry weight LOD: 0.003 mg/kg moist soil, 0.004 mg/kg soil dry weight

The following figure shows a representative chromatogram.

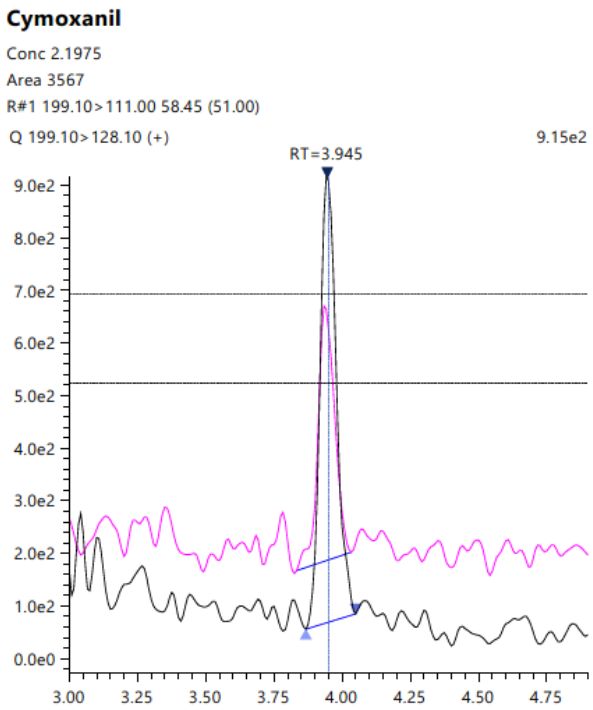


Figure A 82: Chromatogram of cymoxanil

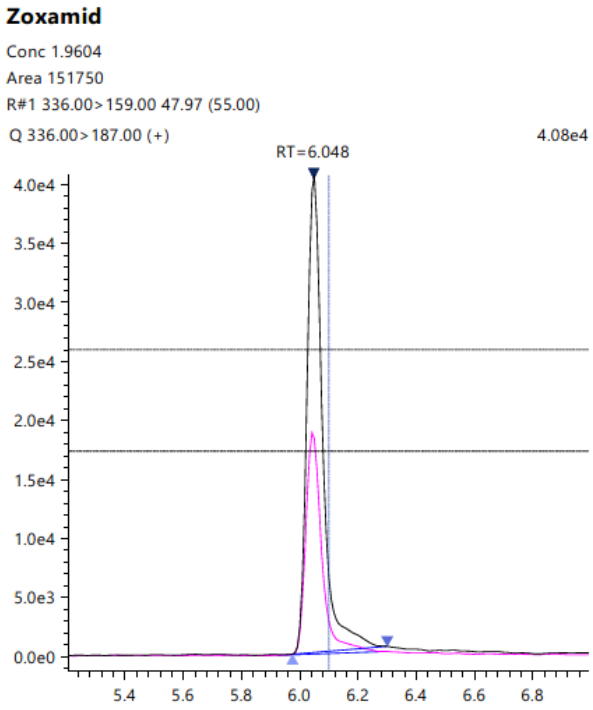


Figure A 83: Chromatogram of zoxamide

Conclusion

A HLPC method with MS/MS detection for the determination of cymoxanil and zoxamide in soil was fully validated according to SANCO/3029/99 rev.4. With this method, cymoxanil and zoxamide can be reliably determined in moist soil from 0.01 to 1.0 mg/kg (0.0125 to 1.25 mg/kg dry weight.

(Thomas H. 2020)

A 2.2.1.2.2 Analytical method 2

A 2.2.1.2.2.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/57**
Report	Friedrich, S., 2020: Effects of Cymoxanil 33 % + Zoxamide 33 % WG on the reproduction of the earthworm *Eisenia andrei* in artificial soil Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No.17 48 TEC 0008, 18 35 CRX 0029, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (report no. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (report no. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (10 % peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with 5 replicates per fortification level at test item at concentrations of approximately 50% of the lowest test concentration (0.55 mg a.i./kg moist soil or 0.74 mg a.i./kg soil d.w.) and approximately 120% of the highest test concentration (38 mg a.i./kg moist soil or 51 mg a.i./kg soil d.w.). Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed.

The analytes were determined in the diluted extraction samples with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 →111), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Shimadzu LC-20 system with an 8040 triple quadrupole mass spectrometric detector
Column:	ACE Excel 3 C18-AR, 100 * 2.1 mm

Mobile phase: A: water containing 0.1 % formic acid and 5 mmol/L ammonium formate
B: methanol containing 0.1 % formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate: 0.4 mL/min
Column temp.: 40°C
Injection volume: 1 µL
Retention time: Cymoxanil: 3.3 - 3.4 min
Zoxamide: 5.4 - 5.5 min
Run time: 9.0 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Cymoxanil:
m/z 199.1 → m/z 128.1 (quantifier ion)
m/z 199.1 → m/z 111.0 (qualifier ion)
Zoxamide:
m/z 336 → m/z 186.95 (quantifier ion)
m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 192: Analysis results in the soil specimens (based on soil dry weight)

Treatment group	Nominal concentration [mg/kg dw]	Day 0		Day 28		Day 56	
		analysed concentration [mg/kg dw]	recovery [%]	analysed concentration [mg/kg dw]	recovery [%]	analysed concentration [mg/kg dw]	recovery [%]
Cymoxanil							
1	0.000	n.d.		n.d.		n.d.	
2	1.509	1.312	87%	0.343 [x]	23	0.129 [x]	8.5
3	2.716	2.449	90%	0.606	22	0.196 [x]	7.2
4	4.889	4.241	87%	1.199	25	0.358 [x]	7.3
5	8.801	7.770	88%	2.282	26	0.759	8.6
6	15.84	14.82	94%	3.681	23	1.076	6.8
7	28.52	26.57	93%	7.585	27	2.062	7.2
8	51.33	46.05	90%	17.960	35	7.487	15
Zoxamide							
1	0.000	n.d.		n.d.		n.d.	

2	1.482	1.200	81	0.394[x]	27	0.143 [x]	10
3	2.667	2.341	88	0.847	32	0.306 [x]	11
4	4.801	4.196	87	1.641	34	0.628 [x]	13
5	8.642	7.673	89	2.857	33	1.289	15
6	15.56	14.61	94	5.298	34	2.184	14
7	28.00	27.52	88	9.839	35	4.007	14
8	50.40	44.98	89	20.844	41	8.874	18

mg/kg dw = mg/kg corrected to soil dry weight
n.d. = not detected
[x] = result below the LOQ validated in this phase

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation is shown in the table below.

Table A 193: Re-validation results

Validation	Replicates	Sample preparation factor [mL/g]	Nominal conc. [mg/kg]	Mean analysed conc. [mg/kg]	Mean recovery [%]	RSD [%]
Cymoxanil (concentration in moist soil)						
Low	5	40	0.559	0.468	84	2.9
High	5	200	38.24	33.82	88	1.0
Zoxamide (concentration in moist soil)						
Low	5	40	0.549	0.485	88	2.4
High	5	200	37.55	34.78	93	1.6
Cymoxanil (concentration in dry soil)						
Low	5	40	0.754	0.632	84	2.9
High	5	200	51.63	45.66	88	1.0
Zoxamide (concentration in dry soil)						
Low	5	40	0.741	0.655	88	2.4
High	5	200	50.69	46.96	93	1.6

Linearity

Linearity was demonstrated for solvent calibration curves (6 concentration levels) of zoxamide in the range of 11.00 – 224.55 µg/L and cymoxanil in the range of 11.27 – 230.01 µg/L. This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 0.56 mg/kg cymoxanil and 0.55 mg/kg zoxamide in moist soil specimens, equivalent to 0.75 mg/kg cymoxanil and 0.74 mg/kg zoxamide in dry weight soil and 14 µg/L

cymoxanil and 14 µg/L zoxamide in diluted extracts. However, the LOQ of the original method (report no. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Limit of detection

The limit of detection (LOD) was defined in the context of this phase of the study as the lowest calibration level, i.e. 11.00 µg/L of zoxamide and 11.27 µg/L of cymoxanil, equivalent to 0.45 mg/kg cymoxanil and 0.44 mg/kg zoxamide in moist soil specimens, equivalent to 0.61 mg/kg cymoxanil and 0.60 mg/kg zoxamide in dry weight soil

Matrix effects

Matrix effects were assessed by evaluating the recovery results from spiked and unspiked control samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, the analytes showed characteristic retention times and interfering peaks in control samples, which eluted at the same retention time as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for at max. 23 hours in the refrigerator. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated

Table A 194: Characteristics of the analytical method used for determination of zoxamide and cymoxanil in artificial soil

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point calibration with external standard. Individual calibration data presented in section 7.1. The calibration was linear. Calibration curve equation: $y = 9473.322\ x + 21465.57$, $r^2=0.99945$	6-point calibration with external standard. Individual calibration data presented in section 7.1. The calibration was linear. Calibration curve equation: $y = 9154.537\ x – 5734.254$, $r^2=0.99996$
Calibration range	11.00 to 224.55 µg/L in the diluted soil extract (xx to yy mg a.s./kg moist soil and xx to yy mg a.s./kg dry soil)	11.27 to 230.01 µg/L in the diluted soil extract (xx to yy mg a.s./kg moist soil and xx to yy mg a.s./kg dry soil)

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Assessment of matrix effects	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of quantification (LOQ)	0.549 mg a.s./kg moist soil (as received), corresponodig to 0.741 mg a.s./kg dry soil and 13.71 µg a.s./L in the analytical sample.	0.559 mg a.s./kg moist soil (as received), corresponodig to 0.754 mg a.s./kg dry soil and 13.97 µg a.s./L in the analytical sample.

The following figure shows a representative chromatogram.

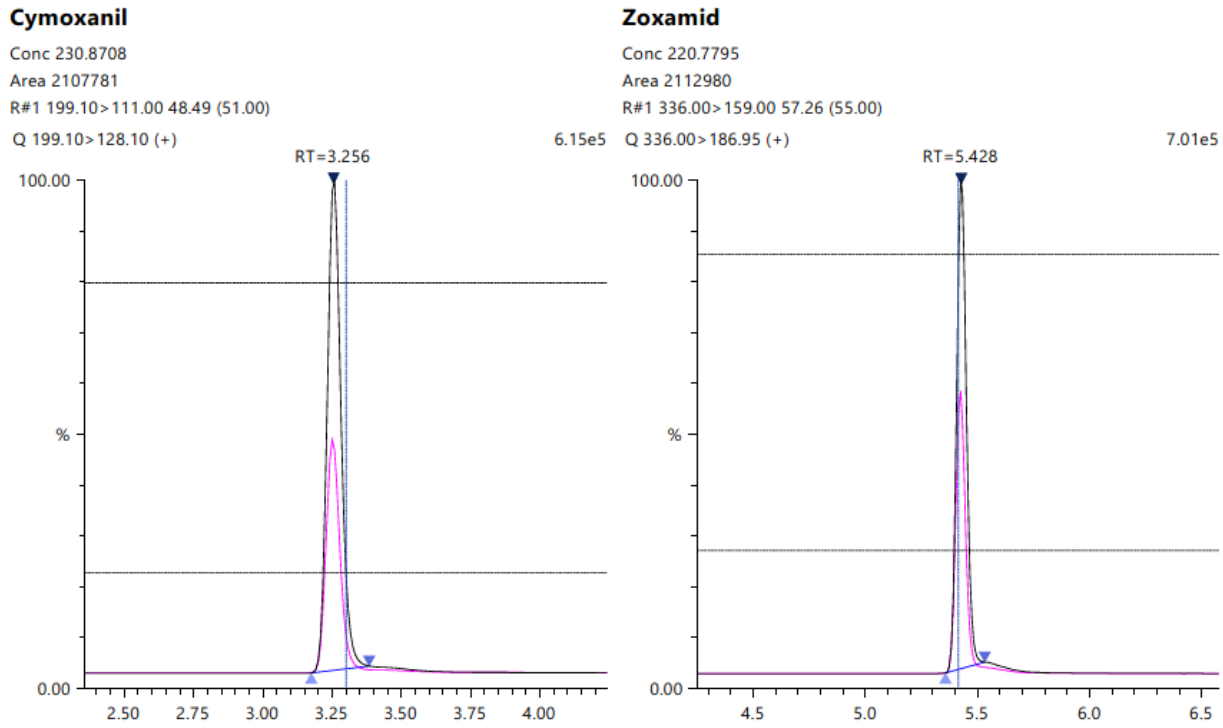


Figure A 84: Chromatograms of the highest calibration standard in 50% methanol containing 0.1% formic acid

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (87-94% for cymoxanil and 81-94% for zoxamide). After 28 days, the cymoxanil concentrations had declined to 22 – 35% of nominal, the zoxamide concentrations to 27 – 41% of nominal. After 56 days, the cymoxanil concentrations had declined to 7 – 15% of nominal, the zoxamide concentrations to 10 – 18% of nominal.

(Friedrich S. 2020)

A 2.2.1.2.3 Analytical method 3

A 2.2.1.2.3.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/58**
Report	Schulz, L., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on earthworms under field conditions Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No.19 48 FEW 0003, 19 35 CRX 0030, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	Volume of acetonitrile used for extraction: 10 mL acetonitrile instead of 20 mL were used for extraction of the soil samples. This has no impact on the outcome of the analytical phase.
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the analytical verification of the concentrations of zoxamide and cymoxanil in field soil and spray targets. The determination was conducted by a validated method, and was re-validated in this study according to SANCO/3029/99 rev.4. In addition, the isomer ratio of (R)- and (S)-zoxamide was determined in selected samples to confirm the chiral stability of zoxamide (racemate).

10 g soil were wetted with 2 mL water and extracted with 10 mL acetonitrile and 0.2 mL acetic acid for 15 minutes on a vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4:1 (w/w) mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were analysed.

Two mass transitions of each analyte were used for detection (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 → 111), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Shimadzu LC-20ADXRsystem with an LCMS-8040mass spectrometric detector
Column:	ACE Excel 3 C18-AR, 100 * 2.1 mm
Mobile phase:	A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate:	0.4 mL/min
Column temp.:	40°C
Run time:	9.0 min
Ionisation:	ESI (electrospray ionisation) positive, MRM
Ion mode	Cymoxanil: m/z 199.1 → m/z 128.1 (quantifier ion) m/z 199.1 → m/z 111.0 (qualifier ion) Zoxamide: m/z 336 → m/z 186.95 (quantifier ion) m/z 336 → m/z 159 (qualifier ion)

HPLC parameters for chiral separation of zoxamide stereoisomers

Instrument:	Shimadzu LC-20ADXRsystem with an LCMS-8040mass spectrometric detector
Column:	Phenomenex Lux Cellulose-3, 150 * 2 mm, 3 µm
Mobile phase:	A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	40	60
8.00	20	80
8.01	0	100
10.00	0	100
10.01	40	60

Flow rate:	0.4 mL/min
Run time:	12.5 min
Ionisation:	ESI (electrospray ionisation) positive, MRM
Ion mode	Zoxamide: m/z 336 → m/z 187 (quantifier ion) m/z 336 → m/z 159 (qualifier ion)

Results and discussions

Recovery findings

Table A 195: Re-validation results for Cymoxanil

Validation level	Nominal concentration mg/kg d.w.	Analysed concentrations mg/kg d.w.	Mean analysed concentration mg/kg d.w.	Mean recovery [%]	RSD [%]
Low (quantifier transition)	0.0118	0.0093, 0.0093, 0.0095, 0.0098, 0.0095	0.0095	80	2.2
High (quantifier transition)	2.350	2.091, 2.035, 2.100, 2.064, 1.947	2.047	87	3.0
Low (qualifier transition)	0.0118	0.0085, 0.0080, 0.0105, 0.0089, 0.0094	0.0090	76	10.5
High (qualifier transition)	2.350	2.115, 2.043, 2.103, 2.105, 1.967	2.067	88	3.0

Table A 196: Re-validation results for zoxamide

Validation level	Nominal concentration mg/kg d.w.	Analysed concentrations mg/kg d.w.	Mean analysed concentration mg/kg d.w.	Mean recovery [%]	RSD [%]
Low (quantifier transition)	0.0118	0.0086, 0.0086, 0.0082, 0.0084, 0.0085	0.0084	71	1.7
High (quantifier transition)	2.339	2.040, 2.020, 2.058, 1.994, 2.055	2.033	87	1.3
Low (qualifier transition)	0.0118	0.0088, 0.0085, 0.0083, 0.0083, 0.0086	0.0085	78	2.4
High (qualifier transition)	2.339	2.055, 2.024, 2.076, 2.042, 2.073	2.054	88	1.1

Accuracy and precision / repeatability

The accuracies, reported as mean recovery and precision / repeatability as relative standard deviation are shown in table 1 above. Mean recoveries for each level are in the range 70-110%, the RSD is < 20% per level. The results fulfill the criteria of SANCO/3029/99 rev.4.

Linearity

Linearity was demonstrated for matrix-matched calibrations (8 concentration levels) in the range of 4 to approximately 1200 µg/L a.i. in soil extract for both analytes, with a correlation coefficient (r) greater than 0.99. This is equivalent to 0.005 to 2.8 mg/kg in dry soil (highest validation concentration diluted 2x).

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e., 0.012 mg/kg zoxamide and cymoxanil, per soil dry weight.

Limit of detection

The limit of detection, calculated as three times the noise / blank response, was 0.0009 mg/kg d.w. for zoxamide and 0.0015 mg/kg d.w. for cymoxanil.

Matrix effects

Matrix-matched calibration was used to compensate possible matrix effects.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two mass transitions per analyte for quantification and confirmation. No interfering peaks >30% of LOQ were detected for both mass transitions.

Storage stability of sample extracts and solvent standards

The max. storage period of sample extracts and standard solution was 4 days. Recovery experiments with spiked extract matrix demonstrated storage stability of sample extracts and standard solutions for at least 4 days at 4-8°C.

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 595 days. The freezer storage stability of zoxamide and cymoxanil residues in soil samples was confirmed for 595 days.

Table A 197: Characteristics of the analytical method used for determination of zoxamide and cymoxanil in artificial soil

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	8-point calibration with external standard. Individual calibration data presented in the report The calibration was linear over the whole calibration range. Calibration curve equation: $y = 93766.3 x + 1178426$, $r^2 = 0.998$	8-point calibration with external standard. Individual calibration data presented in the report The calibration was linear over the whole calibration range. Calibration curve equation: $y = 2427.85 x + 1421.28$, $r^2 = 0.998$
Calibration range	3.98 to 1206 µg/L zoxamide in soil extract (0.005 - 1.43 mg/kg dry soil)	4.00 to 1211 µg/L cymoxanil in soil extract (0.005 – 1.43 mg/kg dry soil)
Assessment of matrix effects	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of quantification (LOQ)	0.012 mg/kg dry soil	0.012 mg/kg dry soil
Limit of detection (LOD)	0.0009 mg/kg dry weight (< 30% of LOQ)	0.0015 mg/kg dry weight (<30% of LOQ)

The following figure shows a representative chromatogram.

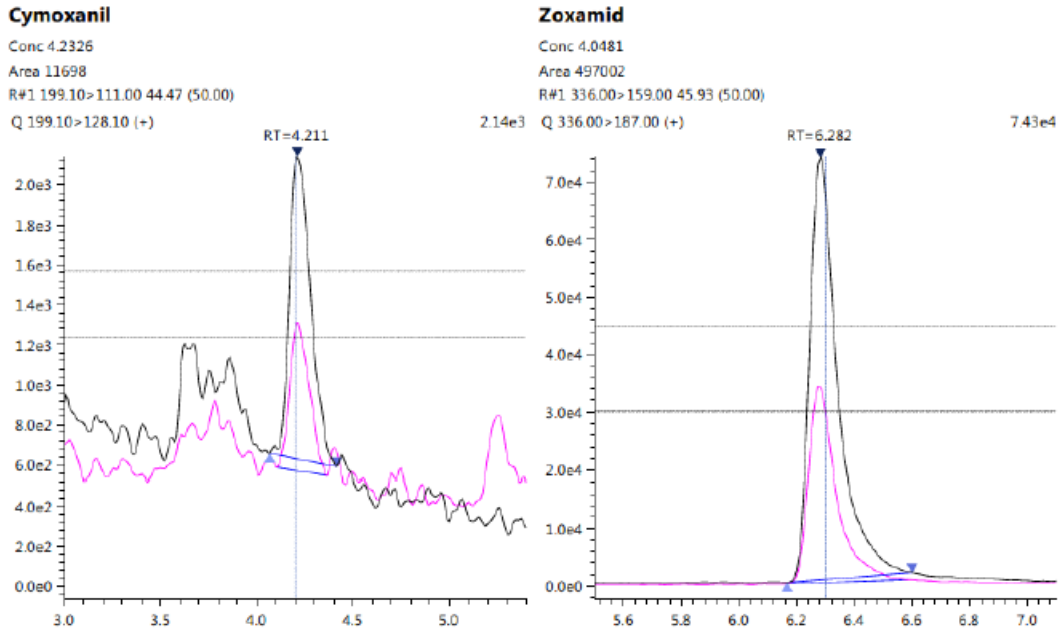


Figure A 85: Chromatogram of the lowest matrix standard

Conclusion

The recoveries in the spray targets (plastic cups filled with 500 g dry soil) were between 74% and 126% after each application. The recoveries in the 0-5 cm soil layer after the first application were 102% and 84% for zoxamide and 101% and 79% for cymoxanil.

The concentrations in the 5 – 20 cm layer were low for zoxamide (maximum mean concentration 0.024 mg/kg in the high treatment after the 5th application) and non-detectable for cymoxanil.

(Schulz L. 2020)

A 2.2.1.2.4 Analytical method 4

A 2.2.1.2.4.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/59**
Report	Parsons, Ch., 2020: Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-3, 18 35 CRX 0026, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of BioChem agrar was used.

Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.

Acceptability: Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (BioChem project No. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with test item at concentrations of 129 mg/kg a.i. in moist soil (161 mg/kg a.i. or 500 mg/kg test item in dry soil) and at 258 mg/kg a.i. in moist soil (323 mg/kg a.i. or 1000 mg/kg test item in dry soil) with 5 replicates per fortification level. This amounts to a working range of approximately 50 - 100% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) was placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the addition of about 2.5 g of a 4 : 1 mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed. The analytes were determined after extraction with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 → 111), one for quantification and one for qualification, respectively.

Equipment

HPLC System: A Shimadzu system with a triple quadrupole mass spectrometric detector was used

Column: ACE Excel3 C18-AR 100 * 2.1 mm, 3 µm

Mobile phase: A: water containing 1mL/L formic acid and 5 mmol/l ammonium formate
B: methanol containing 1 mL/L formic acid and 5 mmol/l ammonium formate

Time [min]	A [%]	B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate: 0.4 mL/min

Retention time: Cymoxanil approx. 3.2 min
Zoxamide approx. 5.4 min

Run time: 9.0 min

Detection: ESI positive, MRM

Ion mode Cymoxanil m/z 199.1 → 128.1, 199.1 → 111.0
Zoxamide m/z 336 → 187, 336 → 159

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 198: Analysis results in moist soil

Treatment group, analyte	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	10000	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	10000	<LOQ		<LOQ		<LOQ	
A, cymoxanil	265.6	1000	270.1	102	222.9	84	193.3	73
A, zoxamide	260.8	1000	268.4	103	262.8	101	250.2	96

n.d.: not detected; <LOQ: concentration below validated limit of quantification (129 mg/kg d.w.), areas of detected peaks were actually below 1.5% of the LOQ (equivalent to approx. 2 mg/kg)

Table A 199: Analysis results in dry soil

Treatment group, analyte	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	<LOQ		<LOQ		<LOQ	
A, cymoxanil	332.0	337.6	102	278.6	84	241.7	73
A, zoxamide	326.0	335.5	103	328.5	101	312.8	96

n.d.: not detected; <LOQ: concentration below validated limit of quantification (161 mg/kg d.w.), areas of detected peaks were actually below 1.5% of the LOQ (equivalent to approx. 2.5 mg/kg)

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the tables below.

Table A 200: Re-validation results for cymoxanil

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.2	120.4	93	4.8
High	5	10000	258.4	238.8	92	9.6
Concentration in dry soil						
Low	5	12500	161.5	150.5	93	4.8
High	5	12500	322.9	298.6	92	9.6

Table A 201: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean	Mean recovery [% of nominal]	RSD [%]

				analysed concentration [mg/kg]		
Concentration in moist soil						
Low	5	10000	129.1	115.9	90	4.3
High	5	10000	258.3	227.7	88	9.0
Concentration in dry soil						
Low	5	12500	161.4	144.9	90	4.3
High	5	12500	322.8	284.6	88	9.0

Linearity

Linearity was demonstrated for solvent calibration curves (5 concentration levels) of zoxamide in the range of 9.6 to 32 µg/L (corresponding to 96 - 320 mg/kg a.s. in moist soil and 120 - 400 mg/kg a.s. in dry soil) and cymoxanil in the range of 9.7 to 32.5 µg/L (corresponding to 97 - 325 mg/kg a.s. in moist soil and 122 - 406 mg/kg a.s. in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 129 mg/kg cymoxanil and zoxamide, respectively, in moist soil, equivalent to 161 mg/kg in dry soil and 13 µg/L in diluted extracts. However, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 1.5 % of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen soil samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated.

Table A 202: Characteristics for the analytical method

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 28970.8x + 27549.4$, $r^2=0.9997$	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 9690.17x + 4540.47$, $r^2=0.9988$
Calibration range	9.6 to 32.0 µg/L in analytical samples (96 to 320 mg/kg in moist soil)	9.7 to 32.5 µg/L in analytical samples (97 to 325 mg/kg in moist soil)
Assessment of matrix effects is presented	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. Validation blank samples had no peaks with areas >1% of the lowest validation samples).	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. No peaks were found in validation blank samples.
Limit of quantification (LOQ)	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.

The following figures show a representative chromatogram.

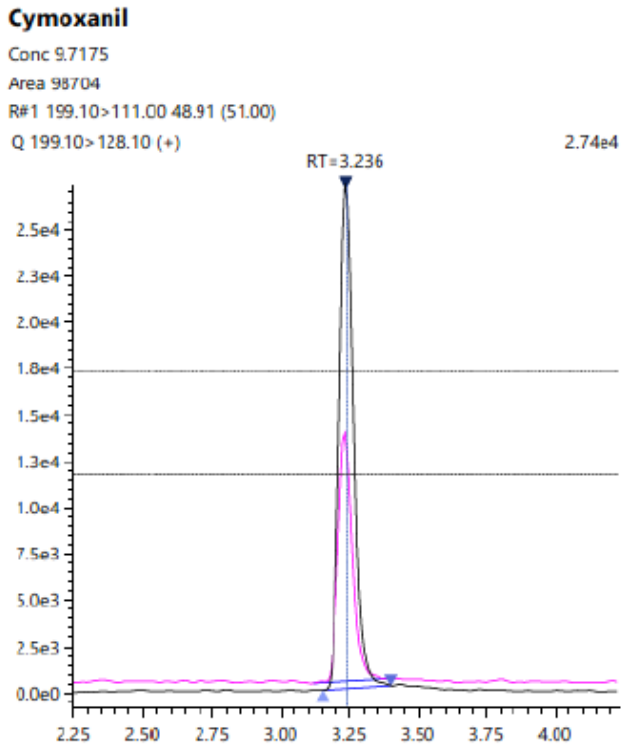


Figure A 86: Chromatogram of cymoxanil

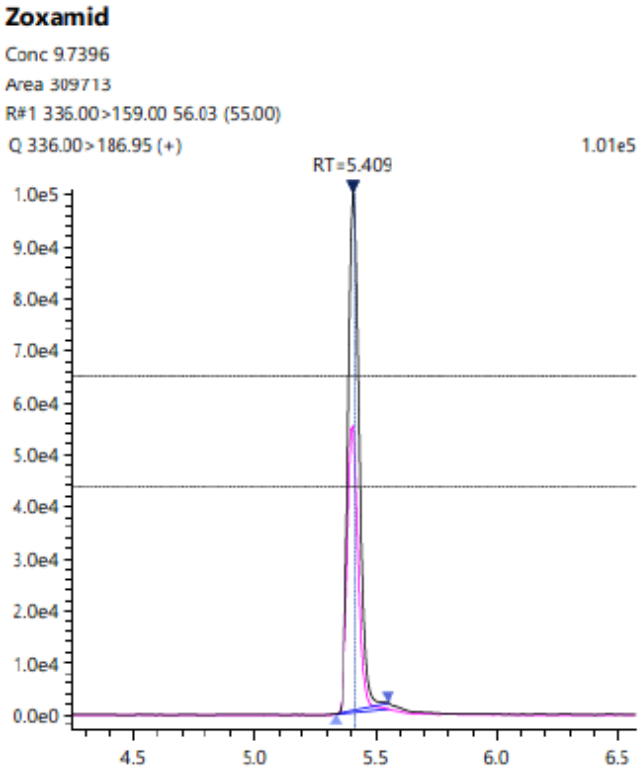


Figure A 87: Chromatogram of zoxamide

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (102% for cymoxanil and 103% for zoxamide). After 28 days, the cymoxanil concentration had declined to 73% of nominal. The zoxamide concentrations stayed within the range of 96 – 103% of nominal throughout the whole study period.

(Parsons Ch. 2020)

A 2.2.1.2.5 Analytical method 5

A 2.2.1.2.5.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/60**
Report	Parson, Ch., 2020: Cymoxanil 33% + Zoxamide 33 % WG (GWN-9823) – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A, Italy Mambo-Tox Ltd., UK, Report No. GOW-17-4; 18 35 CRX 0027, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of BioChem agrar was used. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredients cymoxanil and zoxamide were analysed by a validated method (BioChem project No. 18 35 CRX 0033) using extraction in acetic acetonitrile, separation by reverse-phase high pressure liquid chromatography (HPLC) and tandem mass (MS/MS) determination of the analytes with external standards. Actually, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg soil d.w. for cymoxanil and zoxamide, respectively. However, the method was adapted to the expected concentration range of this study. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3, wetted to 20% water content) spiked with test item at concentrations of 129 mg/kg a.i.in moist soil (161 mg/kg a.i. or 500 mg/kg test item in dry soil) and at 258 mg/kg a.i. in moist soil (323 mg/kg a.i. or 1000 mg/kg test item in dry soil) with 5 replicates per fortification level. This amounts to a working range of approximately 50 - 100% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

10 g soil (weighed to ±0.01 g) were placed in a centrifuge tube and 2 mL water were added. After about 15 minutes swelling, 20 mL acetonitrile, 0.2 mL acetic acid and a ceramic rod were added and the samples were extracted for 15 minutes on a multi-tube vortex shaker. The acetonitrile phase was separated by the

addition of about 2.5 g of a 4:1 mixture of dried magnesium sulfate and sodium chloride and centrifugation. Aliquots of the acetonitrile phase were diluted with a 50% methanol solution containing 0.1% formic acid to the validated concentration range and analysed. The analytes were determined after extraction with two mass transitions each (zoxamide: m/z 336 → 187 and 336 → 159; cymoxanil: m/z 199 → 128 and 199 → 111), one for quantification and one for qualification, respectively.

Equipment

HPLC System:	A Shimadzu system with a triple quadrupole mass spectrometric detector was used
Column:	ACE Excel3 C18-AR 100 * 2.1 mm, 3 µm
Mobile phase:	A: water containing 1mL/L formic acid and 5 mmol/l ammonium formate B: methanol containing 1 mL/L formic acid and 5 mmol/l ammonium formate

Time [min]	A [%]	B [%]
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25

Flow rate:	0.4 mL/min
Retention time:	Cymoxanil approx. 3.2 min Zoxamide approx. 5.4 min
Run time:	9.0 min
Detection:	ESI positive, MRM
Ion mode	Cymoxanil m/z 199.1 → 128.1, 199.1 → 111.0 Zoxamide m/z 336 → 187, 336 → 159

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Recovery findings

Table A 203: Analysis results in moist soil

Treatment group, analyte	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control, cymoxanil	0.0	10000	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	10000	<LOQ		<LOQ		<LOQ	
A, cymoxanil	265.6	1000	230.9	87	216.8	82	202.5	76
A, zoxamide	260.8	1000	233.7	90	246.4	94	244.3	94

n.d.: not detected; <LOQ: concentration below validated limit of quantification (129 mg/kg d.w.), areas of detected peaks were actually below 1% of the LOQ (equivalent to approx. 1.3 mg/kg)

Table A 204: Analysis results in dry soil

Treatment group, analyte	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]

Control, cymoxanil	0.0	n.d.		n.d.		n.d.	
Control, zoxamide	0.0	<LOQ		<LOQ		<LOQ	
A, cymoxanil	332.0	288.6	87	271.0	82	253.1	76
A, zoxamide	326.0	292.1	90	308.0	94	305.4	94

n.d.: not detected; <LOQ: concentration below validated limit of quantification (161 mg/kg d.w.), areas of detected peaks were actually below 1% of the LOQ (equivalent to approx. 2 mg/kg)

Accuracy and precision / repeatability

The accuracies, reported as mean recovery ± relative standard deviation are shown in the tables below.

Table A 205: Re-validation results for cymoxanil

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.2	120.4	93	4.8
High	5	10000	258.4	238.8	92	9.6
Concentration in dry soil						
Low	5	12500	161.5	150.5	93	4.8
High	5	12500	322.9	298.6	92	9.6

Table A 206: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	10000	129.1	115.9	90	4.3
High	5	10000	258.3	227.7	88	9.0
Concentration in dry soil						
Low	5	12500	161.4	144.9	90	4.3
High	5	12500	322.8	284.6	88	9.0

Linearity

Linearity was demonstrated for solvent calibration curves (5 concentration levels) of zoxamide in the range of 9.6 to 32 µg/L (corresponding to 96 - 320 mg/kg a.s. in moist soil and 120 - 400 mg/kg a.s. in dry soil) and cymoxanil in the range of 9.7 to 32.5 µg/L (corresponding to 97 - 325 mg/kg a.s. in moist soil and 122 - 406 mg/kg a.s. in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 129 mg/kg cymoxanil and zoxamide, respectively, in moist soil, equivalent to 161 mg/kg in dry soil and 13 µg/L in diluted extracts. However, the LOQ of the original method (BioChem project No. 18 35 CRX 0033) is 0.01 mg/kg dry soil for cymoxanil and zoxamide, respectively.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide and cymoxanil in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Storage stability of frozen soil samples

Since the nominal concentrations were recovered in the day 0 sample, stability is demonstrated.

Table A 207: Characteristics for the analytical method

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 28970.8x + 27549.4$, $r^2=0.9997$	5-point calibration with external standard. The calibration was linear. Calibration curve equation: $y = 9690.17x + 4540.47$, $r^2=0.9988$
Calibration range	9.6 to 32.0 µg/L in analytical samples (96 to 320 mg/kg in moist soil)	9.7 to 32.5 µg/L in analytical samples (97 to 325 mg/kg in moist soil)
Assessment of matrix effects is presented	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. Validation blank samples had no peaks with areas >1% of the lowest validation samples).	Because of the high dilution of soil extracts, matrix effects can be excluded. This is proven by recoveries of validation samples within 70-110%. No interfering peaks were observed. No peaks were found in validation blank samples.
Limit of quantification (LOQ)	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.	129 mg/kg a.i. in moist soil (as received), corresponding to 161 mg/kg a.i. in dry soil and 13 µg/L a.i. in the analytical sample.

The following figures show a representative chromatogram.

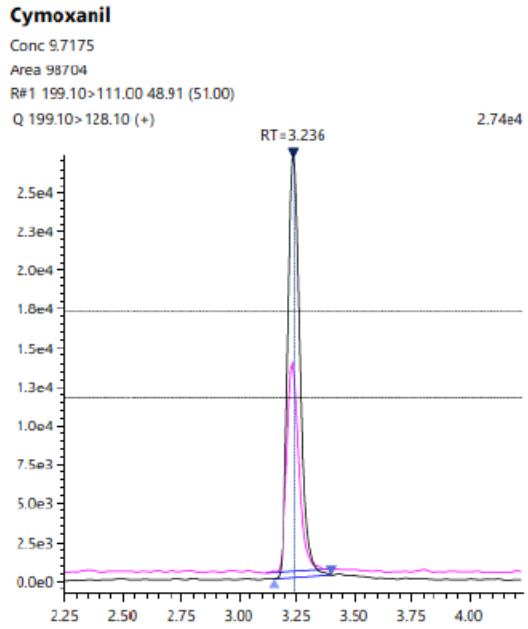


Figure A 88: Chromatogram of cymoxanil

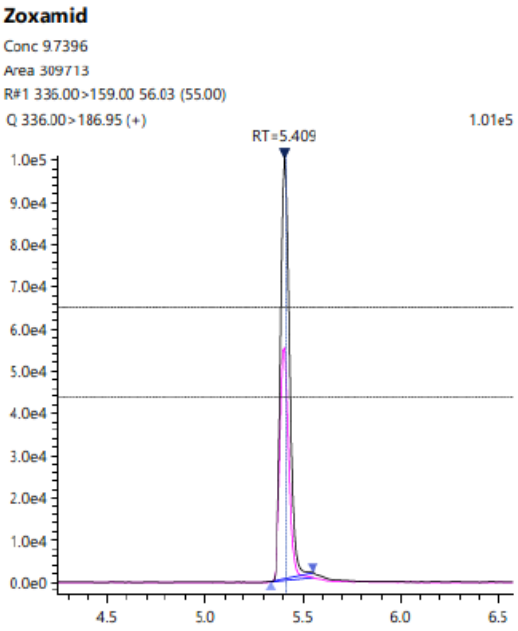


Figure A 89: Chromatogram of zoxamide

Conclusion

The nominal initial concentrations in the fresh soil specimens could be confirmed – the recoveries were all greater than 80% (87% for cymoxanil and 90% for zoxamide). After 14 days, the cymoxanil concentration had declined to 76% of nominal. The zoxamide concentrations stayed within the range of 90 – 94% of nominal throughout the whole study period.

(Parson Ch. 2020)

A 2.2.1.3 Description of analytical method for the determination of residues in bees

and bee larvae

A 2.2.1.3.1 Analytical method 1

A 2.2.1.3.1.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/75**
Report	Ruhland, S., 2018: Chronic toxicity of Cymoxanil 33% + Zoxamide 33% WG to the honey bee *Apis mellifera* L. under laboratory conditions Gowan Crop Protection Ltd., UK, Oxon Italia S.p.A, Italy BioChem agrar, Germany, Report No. 17 48 BAC 0005, 17 35 CRB 0009, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the verification of the concentration of the active ingredients cymoxanil and zoxamide in the test item feeding solutions. The determination was conducted by an in-house developed method using high performance liquid chromatography (HPLC) with UV-detection.

Determination was performed by high pressure liquid chromatography (HPLC) with UV-detection (UV). The specificity of the method was assured by continuously recorded UV spectra from 200 to 300 nm with a diode-array detector (DAD). Spectra of the test item peaks were compared to those of the references. As a result, similar spectra with approximately equal absorption maxima, constant chromatographic retention times and no interfering peaks were observed.

The method fulfils all criteria of the guidance document SANCO/3029/99 for the determination of both active substances:

– blank values do not exceed 30% of the lowest validated concentration,
– mean recoveries for each level are in the range 70-110%,
– the RSD is < 20% per level.

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 46.40 mg/L of cymoxanil and 46.69 mg/L of zoxamide.

No confirmatory method or ILV is required, since the method is used for pre-registration purposes only. The method was validated specifically for the present study.

Equipment

Instrument: Shimadzu HPLC system with a triple quadrupole mass spectrometric detector

Column: Macherey Nagel Nucleoshell RP18, 2.0 mm x 100 mm, 2.7 µm

Mobile phase: A: Water with 0.1% (v/v) acetic acid + 10% (v/v) B
B: Acetonitrile with 33% (v/v) MeOH and 0.1% (v/v) acetic acid

Gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0.00 min	95	5
8.00 min	5	95
10.00 min	5	95
10.01 min	95	5
12.00 min	Stop	Stop

Flow rate: 0.35 mL/min

Detection: UV-detection at 240 nm for cymoxanil
UV-detection at 211 nm for zoxamide

Retention time: Approx. 3.5 min for cymoxanil
Approx. 7.5 min for zoxamide

Results and discussions

Recovery findings

Table A 208: Recovery results from method validation of analytes using the analytical method

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Test medium*	Cymoxanil	46.40	97	0.2	None
Test medium*	Cymoxanil	1542	98	0.6	None
Test medium*	Zoxamide	46.69	88	0.6	None
Test medium*	Zoxamide	1551	85	6.3	None

* 50% (w/v) sucrose solution containing 0.1% (w/v) xanthan

Accuracyand precision / repeatability

Accuracy was tested by spiking sample matrix with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%.

Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level.

Linearity

Linearity was tested for 5 points at a concentration range of at least ± 20% of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 46.40 mg/L of cymoxanil and 46.69 mg/L of zoxamide.

Matrix effects

Recoveries of validation samples were within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.

Specificity

The specificity of the method was assured by the following method: UV spectra from 200 to 300 nm were continuously recorded by the diode-array detector. Spectra of the peaks were compared to those of the reference. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.

Stability of sample extracts

The maximum storage time of deep-frozen feeding solutions was < 30 days. Extracts for analysis were stored for < 24 hours in the refrigerator.

Table A 209: Characteristics for the analytical method used for validation zoxamide and cymoxanil residues in bees

	Zoxamide	Cymoxanil
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks
Calibration (type, number of data points)	5-point calibration with external standard Linear calibration, no weighting was used. Calibration curve equation: $y = 114408 x – 8153.70$, $r^2 = 0.9999827$	5-point calibration with external standard Linear calibration, no weighting was used. Calibration curve equation: $y = 45044.9 x – 2379.70$, $r^2 = 0.9999935$
Calibration range	4.555 to 22.77 mg/L in analytical samples	4.344 to 21.72 mg/L in analytical samples
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 46.69 mg/L (regarding dilution factor 7.470 mg/L) LOD:	46.40 mg/L (regarding dilution factor 7.425 mg/L) LOD:

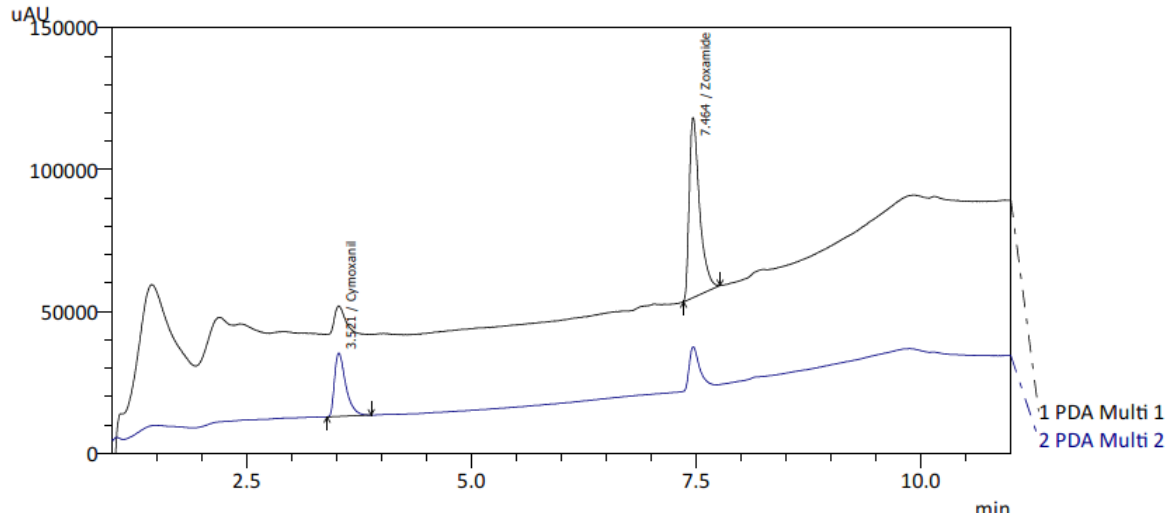


Figure A 90: **Chromatogram of cymoxanil and zoxamide**

Conclusion

A method for pre-registration purposes was successfully validated according to SANCO/3029/99 rev. 4 and regarded acceptable for the determination of zoxamide and cymoxanil in the test item feeding solutions. The nominal test item concentrations in the feeding solutions were analytically verified.

(Ruhland S. 2018)

A 2.2.1.3.2 Analytical method 2

A 2.2.1.3.2.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/76**
Report	Scheller, K., 2020: Cymoxanil 33% + Zoxamide 33% WG - Repeated expos re of honey bee (*Apis mellifera* L.) larvae under laboratory conditions (*in vitro*) Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 17 48 BLC 0005, 17 35 CRB 0010, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the verification of the concentration of the active ingredients cymoxanil and zoxamide in the test item stock solutions. The analytical method was validated according to SANCO/3029/99 rev. 4.

Determination was performed by high pressure liquid chromatography (HPLC) with UV-detection (UV). The specificity of the method was assured by continuously recorded UV spectra from 200 to 300 nm with a diode-array detector (DAD). Spectra of the test item peaks were compared to those of the reference items. As a result, similar spectra with approximately equal absorption maxima, constant chromatographic retention times and no interfering peaks were observed.

No confirmatory method is required, since the method is used for pre-registration purposes only. The method was validated specifically for the present study.

The recoveries of cymoxanil in the specimens were between 100% and 105%, the recoveries of zoxamide were between 87% and 91%. No active ingredient was detected in the control specimens. Thus, the test item concentrations in the stock solutions of the feeding solutions from the biological part were analytical verified.

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 8.646 mg/L of cymoxanil and 8.594 mg/L of zoxamide.

Equipment

A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used.

Column: Macherey Nagel Nucleoshell RP18, 2.0 mm x 100 mm, 2.7 µm

Mobile phase: A: Water with 0.1% (v/v) acetic acid + 10% (v/v) B
B: Acetonitrile with 33% (v/v) MeOH and 0.1% (v/v) acetic acid

Gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0.00 min	95	5
8.00 min	5	95
10.00 min	5	95
10.01 min	95	5
12.00 min	Stop	Stop

Flow rate: 0.35 mL/min

Detection: UV-detection at 240 nm for cymoxanil
UV-detection at 211 nm for zoxamide

Retention time: Approx. 3.0 min for cymoxanil
Approx. 7.1 min for zoxamide

Results and discussions

Recovery findings

Table A 210: Recovery results

Matrix	Analyte	Fortification level (mg/L) (n=5)	Mean recovery (%)	RSD (%)	Comments
Test medium*	cymoxanil	8.594	95	3.3	None
Test medium*	cymoxanil	306.7	102	1.0	
Test medium*	zoxamide	8.646	97	2.1	
Test medium*	zoxamide	308.6	84	0.6	

* ASS containing 18% (w/v) glucose, 18% (w/v) fructose, 4% (w/v) yeast extract

Accuracyand precision / repeatability

Accuracy was tested by spiking sample matrix with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%. Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level.

Linearity

Linearity was tested for 5 points at a concentration range of at least ± 20% of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this study as the lowest successfully validated fortification level, i.e. 8.646 mg/L of cymoxanil and 8.594 mg/L of zoxamide.

Matrix effects

Recoveries of validation samples were within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.

Specificity

The specificity of the method was assured by the following method: UV spectra from 200 to 300 nm were continuously recorded by the diode-array detector. Spectra of the peaks were compared to those of the

reference. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.

Stability of sample extracts

The specimens for analysis were stored at ≤ -18 °C. The largest specimen storage was 16 days (≤ 30 days); therefore, storage stability testing was not necessary.

Table A 211: Characteristics for the analytical method used for validation zoxamide residues in bee larvae

	Cymoxanil	Zoxamide
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time of the analyte, no interfering peaks	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time of the analyte, no interfering peaks
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data presented in section 8.1. The calibration was linear, no weighting was used. Calibration curve equation: y = 74965.2 x + 3179.15, r^2 = 0.9999753	5-point calibration with external standard Individual calibration data presented in section 8.1. The calibration was linear, no weighting was used. Calibration curve equation: y = 191327 x – 11410.4, r^2 = 0.9999883
Calibration range	1.690 to 8.448 mg/L in analytical samples	1.847 to 9.233 mg/L in analytical samples
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
Limit of quantification (LOQ) Limit of detection (LOD)	8.646 mg/L (2.767 mg/L if the sample dilution is taken into account)	8.594 mg/L (2.750 mg/L if the sample dilution is taken into account)

The following figure shows a typical chromatogram.

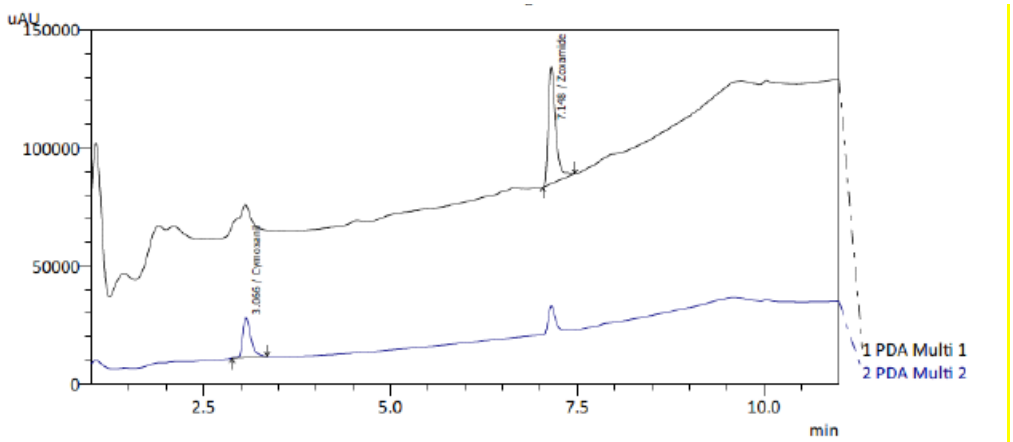


Figure A 91: Chromatogram of cymoxanil and zoxamide

Conclusion

A method for pre-registration purposes was successfully validated according to SANCO/3029/99 rev. 4 and

regarded acceptable for the determination of zoxamide and cymoxanil in the test item feeding solutions. The nominal test item concentrations in the feeding solutions were analytically verified.

(Scheller K. 2020)

A 2.2.1.3.3 Analytical method 3

A 2.2.1.3.3.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/77**
Report	Schnurr, A., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 18 48 BFB 0001, 18 35 CRB 0040, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4 (2000) SANCO/825/00 rev. 8.1 (2010)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of cymoxanil and zoxamide after field application of the test item Cymoxanil 33% + Zoxamide 33% WG.

The analysis of specimens (spray solutions, nectar (forager bees and in-hive), bees (dead and alive bees), pollen (trap and in-hive) and flowers was conducted by an in-house development method based on the QuEChERS extraction method inclusive clean-up of Anastassiades *et al.* (2003) using high performance liquid chromatography (HPLC) with mass-spectrometric (MS-MS) detection of cymoxanil and zoxamide based on the methods of Jooß (2013) and Melkebeke (2000), respectively. However, the new method allows the detection of both active substances in one run.

The method involves the extraction of 1g samples (flowers and bees) with 2.5 mL acetonitrile and 2.5 mL water, followed by liquid/liquid partitioning after addition of 2g anhydrous MgSO4/NaCL (4:1 w/w). In case of the matrix pollen, extraction was done without 2.5 mL water. The sample extracts were cleaned by solid-phase extraction with 50 mg Envi-Carb (GCB), magnesium sulfate, PSA and C-18 (1:1:1:1, w/w/w/w). The sample extracts were diluted with acetonitrile/water/blank extract (25/50/25 v/v/v) containing 0.1% formic acid in a way that the concentration of the diluted extract was within the range of the respective calibration curve. The active substances in the cleaned extracts were separated on a reverse phase column by high performance liquid chromatography (HPLC). They were detected by tandem mass-spectrometry monitoring two mass transitions each (cymoxanil: m/z 199 →128 and 199 → 111), zoxamide: m/z 336 → 187 and 336 → 159) for quantification and/or qualification, respectively. The analysis was performed with external standard solutions (for spray solutions) or matrix-matched standards (for flowers, nectar, bees and pollen). The limit of quantification (LOQ) of the method was set at 0.005 mg/L (for spray solution) and at

0.005 mg/kg (for solid commodity) per analyte. The method was fully validated according to SANC0/3029/99 rev. 4 taking into account additional measures as required in SANC0/825/00 (2010).

Equipment

HPLC system: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector was used

Column: ACE Excel 3 C18-AR, 3µm, 100*2.1 mm

Mobile phase: A: Water containing 0.1% formic acid
B: Methanol containing 0.1% formic acid

Gradient:

Time (min)	A (%)	B (%)
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25
9.00	Stop	Stop

Flow rate: 0.4 mL/min

Run time 9.00 min

Oven Temperature: 40°C

Injection volume: 20µL

Retention time: 3.2 min for cymoxanil
5.4 min for zoxamide

Detection: ESI positive, MRM,
Cymoxanil: m/z 199.10 → 128.10 (quantifier), 199.10 → 111.00 (second quantifier of qualifier)
Zoxamide: m/z: 336.00 → 186.95 (quantifier), 336.00 → 159.00 (second quantifier of qualifier)

Results and discussions

Recovery findings

Table A 212: Recovery results from method validation of the quantifier

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil 199.10 → 128.10 (quantifier)		Zoxamide 336.00 → 186.95 (quantifier)	
				Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	LOQ	5	0.005	102	4-3	92	14.5
	200 x LOQ	5	1.000	97	2-9	89	5.5
	Blank	2	0.000	n.d.	-	n.d.	-
Nectar	LOQ	5/4*	0.005	80	9.9	71	4.2
	200 x LOQ	5	1.000	97	1.6	91	5.3
	Blank	2	0.000	n.d.	-	n.d.	-
Bees	LOQ	4**	0.005	103	3.0	84	6.5
	200 x LOQ	5	1.000	99	1.2	76	1.5
	Blank	2	0.000	n.d.	-	n.d.	-
Pollen	LOQ	4**	0.005	105	12.3	109	7.8
	200 x LOQ	5	1.000	109	2.8	105	3.7

	Blank	2	0.000	n.d.	-	n.d.	-
Flowers	LOQ	5	0.005	109	7.6	100	13.2
	200 x LOQ	5	1.000	108	3.1	109	2.8
	Blank	2	0.000	n.d.	-	n.d.	-

* for cymoxanil 5 replicates were taken into account and for zoxamide 4 replicates were taken into account since one sample was an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
** one sample was not taken into account since it is an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
n.d. = not detected
n = replicates

Table A 213: Recovery results from method validation of the qualifier

Matrix	Validation level	N	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil 199.10 → 111.00 (qualifier)		Zoxamide 336.00 → 159.00 (qualifier)	
				Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	LOQ	5	0.005	104	6.7	96	15.4
	200 x LOQ	5	1.000	98	1.9	89	5.9
	Blank	2	0.000	n.d.	-	n.d.	-
Nectar	LOQ	5/4*	0.005	75	10.7	74	4.6
	200 x LOQ	5	1.000	97	2.0	91	4.2
	Blank	2	0.000	n.d.	-	n.d.	-
Bees	LOQ	4**	0.005	81	16.7	76	7.0
	200 x LOQ	5	1.000	86	6.3	75	1.7
	Blank	2	0.000	n.d.	-	n.d.	-
Pollen	LOQ	4**	0.005	88	13.4	101	13.6
	200 x LOQ	5	1.000	98	4.2	105	4.0
	Blank	2	0.000	n.d.	-	n.d.	-
Flowers	LOQ	5	0.005	84	16.4	94	13.1
	200 x LOQ	5	1.000	107	6.7	109	4.4
	Blank	2	0.000	n.d.	-	n.d.	-

* for cymoxanil 5 replicates were taken into account and for zoxamide 4 replicates were taken into account since one sample was an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
** one sample was not taken into account since it is an outlier ($p < 0.05$ and/or 0.01 according to Grubbs as well as Dixon-test)
n.d. = not detected
n = replicates

Accuracyand precision / repeatability

The recovery values were in the range of 70-110% with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for solvent calibration and all matrix matched calibration curves (6 concentration levels) of cymoxanil in the range of 0.101 – 10.10 µg/L (corresponding to 0.001 – 2.02 mg/kg for solid commodities) and zoxamide in the range of 0.101 – 10.08 µg/L (corresponding to 0.001 – 2.02 mg/kg for solid commodities). This covers ranges from at least 20% of the LOQ to at least 202% above 200 x LOQ, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was 0.005 mg/L (for spray solution) and 0.005 mg/kg (for flowers, nectar, bees, pollen) of cymoxanil and zoxamide (corresponding to 0.499 µg/L of cymoxanil and 0.502 µg/L of zoxamide in diluted samples and samples extract).

Limit of detection

The limit of detection (LOD) was 0.001 mg/L (for spray solution) and 0.001 mg/kg (for flowers, nectar, bees, pollen) of cymoxanil and zoxamide (corresponding to 0.101 µg/L of cymoxanil in diluted samples and samples extract, corresponding to 0.101 µg/L of zoxamide in diluted samples and samples extract).

Matrix effects

Matrix effects > 20% were observed for flowers, nectar, bees and pollen samples. For pollen and bee extracts, a strong suppression with up to approximately 50% was demonstrated. The nectar extracts show a strong enhancement partially with over 300%. The flowers extracts show strong enhancement at low concentration and a slightly suppression at high concentrations. Therefore, matrix-matched standard solutions were used for the solid commodities.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Stability of sample extracts and solvent standards

During the whole experimental phase of 11 days in fridge at 4-10°C, the samples were stable (difference of the mean recoveries: < 20% in 50% methanol containing 0.1% formic aid. The 11 days old storage samples were measured against fresh prepared samples.

Storage stability of frozen samples

Samples were stored for at max. 362 days in the freezer (at ≤ -18°C). Therefore, the freezer storage stability was tested with freshly spiked retain (control) samples analysed in parallel to stored samples and proofed acceptable for both active substances.

Table A 214: Storage stability

Matrix	Sample	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil		Zoxamide	
			Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	Stored	1.000	95	0.3	92	10.2
	Fresh	1.000	96	1.5	92	13.0
	Difference	-	1	-	0	-
Nectar	Stored	1.000	106	1.2	84	0.9
	Fresh	1.000	98	0.7	91	0.8
	Difference	-	8	-	7	-
Bees	Stored	1.000	87	1.4	72	1.5
	Fresh	1.000	97	0.6	78	1.6
	Difference	-	10	-	6	-
Pollen	Stored	1.000	80	0.7	106	13.9
	Fresh	1.000	98	2.1	105	1.0

	Difference	-	18	-	1	-
Flowers	Stored Fresh	1.000	98	11.9	96	16.7
		1.000	108	0.8	108	1.4
	Difference	-	10	-	12	-

Table A 215: Characteristics of the analytical method for spay solution

	Zoxamide	**Cymoxanil**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/solvent calibration. The calibration was linear and weighed 1/c. Calibration curve equation: y = 18331.4 x + 128.725, r^2 = 0.99948	6-point calibraqtion with external standard/solvent calibration. The calibration was linear and weighed 1/c. Calibration curve equation: y = 60463.2 x + 657.923, r^2 = 0.99976
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./L spray solution)*	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./L spray solution)*
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L*, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L*, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

* In case of spray solution: mg/kg = mg/L

Table A 216: Characteristics of the analytical method for nectar

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 20251.5 x + 4572.22, r^2 = 0.99988	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y =57548.2 x + 12781.3, r^2 = 0.99980
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

Table A 217: Characteristics of the analytical method for bees

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (25% v/v extract). The calibration was linear and weighed 1/c. Calibration curve equation: y = 13715.0 x + 923.474, r^2 = 0.99997	6-point calibraqtion with external standard//matrix matched calibration (25% v/v extract). The calibration was linear and weighed 1/c. Calibration curve equation: y =50253.7 x + 4249.81, r^2 = 0.99983
Calibration range	0.101 to 10.1 µg/L in analytical	0.101 to 10.1 µg/L in analytical

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

Table A 218: Characteristics of the analytical method for pollen

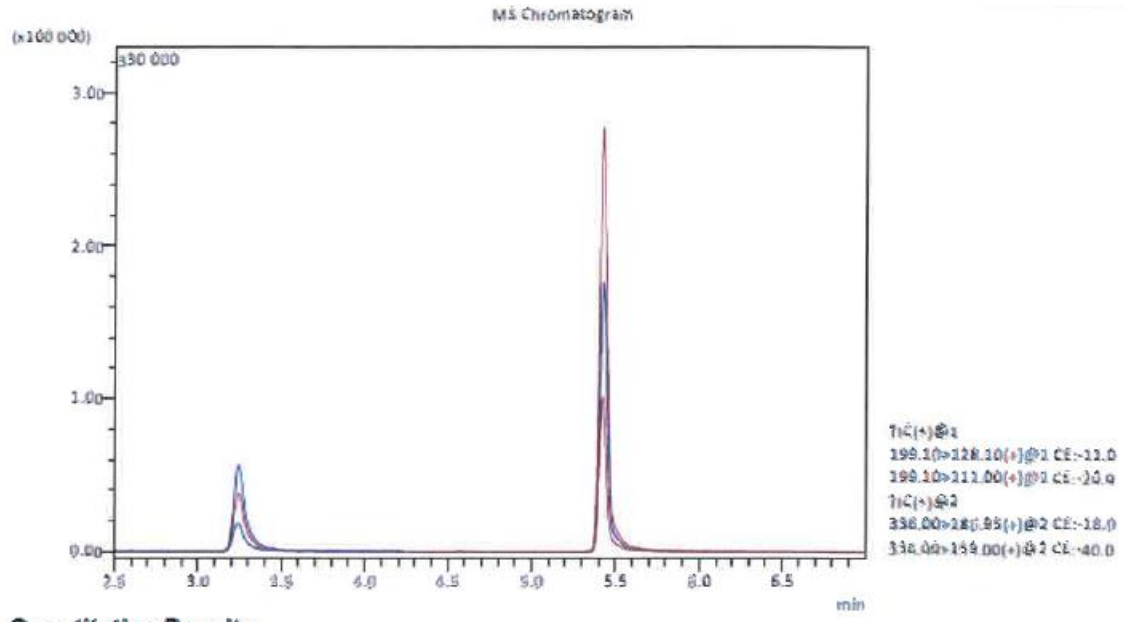
	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 24181.7 x + 1556.20, r^2 = 0.99969	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y =43166.0 x + 2014.47, r^2 = 0.99938
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

Table A 219: Characteristics of the analytical method for flowers

	Zoxamide	Cymoxanil
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	6-point calibraqtion with external standard/matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 20679.9 x + 6396.70, r^2 = 0.99955	6-point calibraqtion with external standard//matrix matched calibration (10% v/v matrix). The calibration was linear and weighed 1/c. Calibration curve equation: y = 34213.2 x + 12077.5, r^2 = 0.99996
Calibration range	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)	0.101 to 10.1 µg/L in analytical samples (corresponding to 0.001 to 2.02 mg a.i./kg nectar)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).	Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks > 30% of the lowest validation samples).
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.005 mg/L, corresponding to 0.499 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample	LOQ: 0.005 mg/L, corresponding to 0.502 µg/L in the analytical sample LOD: 0.001 mg/L, corresponding to 0.101 µg/L in the analytical sample

The following figure shows a typical chromatogram.



Quantitative Results

ID#	Name	Ret. Time	m/z	Area	Height	Conc.	Unit
1	Cymoxanil	3.243	199.10>128.10	184812	37958	10.07469	µg/L
2	Zoxamid	5.426	336.00>186.95	603959	176448	9.97797	µg/L

Figure A 92: Chromatogram of cymoxanil and zoxamide

Conclusion

The method was fully validated on the test matrices spray solution, nectar, bees, pollen and flowers spiked with the test item (Cymoxanil 33% + Zoxamide 33% WG) at LOQ (0.005 mg/kg) and 200x LOQ (1 mg a.i./kg). As a result, all requirements of SANC0/3029/99 rev. 4 and SANC0/825/00 rev 8.1 were fulfilled

(Schnurr A. 2020)

A 2.2.1.3.4 Analytical method 4

A 2.2.1.3.4.1 Method validation

Comments of zRMS:	This study was accepted already in zoxamide section.

Reference:	**KCP 5.1/78**
Report	Schnurr, A., 2020: Effects of Cymoxanil 33% + Zoxamide 33% WG on the honeybee *Apis mellifera* L. under field conditions in Spain (Southern zone) with additional assessments on colony and brood development Gowan Crop Protection Ltd., UK, SIPCAM OXON S.p.A., Italy BioChem agrar, Germany, Report No. 19 48 BFB 0001, 18 35 CRB 0086, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANCO/825/00 rev. 8.1 (2010)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of cymoxanil and zoxamide after field application of the test item Cymoxanil 33% + Zoxamide 33% WG in spray solutions, nectar (forager bees and in-hive), flowers, pollen (trap and in-hive) and bees (dead and alive bees).

Sample preparation of flowers, pollen and bees was based on the QuEChERS extraction method including clean-up of extracts according to Anastassiades et al. (2003). The analyses were carried out using high performance liquid chromatography (HPLC) with mass-spectrometric (MS-MS) detection of cymoxanil and zoxamide based on the methods of Jooß (2013) and Melkebeke (2000). However, the method allows the detection of both active substances in one run.

Sample preparation involves the extraction of 1 g sample (flowers and bees) with 2.5 mL acetonitrile and 2.5 mL water, followed by liquid/liquid partitioning after addition of 2 g anhydrous MgSO4/NaCl (4:1 w/w). In case of the matrix pollen, extraction was done without 2.5 mL water. The sample extracts were cleaned up by dispersive solid-phase extraction using each 50 mg Envi-Carb (GCB), MgSO4, PSA and C-18 (1:1:1:1, w/w/w/w). The sample extracts were diluted with acetonitrile/water/blank extract (25/50/25 v/v/v) containing 0.1% formic acid in a way that the concentration of the diluted extract was within the range of the respective calibration curve. The active substances in the purified extracts were separated on a reversed-phase column by high performance liquid chromatography (HPLC). Detection was carried out by tandem mass-spectrometry, monitoring two mass transitions for each substance (cymoxanil: m/z 199 → 128 and 199 →111; zoxamide: m/z 336 → 187 and 336 → 159) for quantification and/or qualification, respectively. The analysis was performed with external standard solutions (for spray solutions) or matrix-matched external standards (for flowers, nectar, bees and pollen). The limit of quantification (LOQ) of the method was set at 0.005 mg/L (for spray solution) and at 0.005 mg/kg (for solid commodities) per analyte. The method was fully validated according to SANCO/3029/99 rev. 4 taking into account additional measures as required in SANCO/825/00 (2010) (Reference: Biochem project No.: 18 35 CRB 0040, analytical phase to Biochem project No.: 18 48 BFB 0001).

Equipment

Instrument: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector

Column: ACE Excel 3 C_{18}-AR, 3µm, 100*2.1 mm

Mobile phase: A: Water containing 0.1% formic acid
B: Methanol containing 0.1% formic acid

Gradient:

Time (min)	A (%)	B (%)
0.00	75	25
5.00	0	100
7.00	0	100
7.01	75	25
9.00	Stop	Stop

Flow rate: 0.4 mL/min
Run time 9.00 min

Oven Temperature: 40°C

Injection volume: 20µL
40µL (bee specimens)

Retention time: Cymoxanil: 3.3 min
Zoxamide: 5.4 min

Detection: ESI positive, MRM,
Cymoxanil: m/z 199.10 → 128.10 (quantifier), 199.10 → 111.00 (second quantifier of qualifier)
Zoxamide: m/z: 336.00 → 186.95 (quantifier), 336.00 → 159.00 (second quantifier of qualifier)

Results and discussions

Recovery findings

Table A 220: Recovery results from method verification (quantifier)

Matrix	Validation Level	N	Fortification level [mg/kg] (for spray solution [mg/L])		Cymoxanil 199.1 → 128.1 (quantifier)		Zoxamide 336.00 → 186.9 (quantifier)	
			Cymoxanil	Zoxamide	Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	High	5	767.71	771.46	103	2.0	107	10.4
	200x LOQ	3	1.075	1.080	104	2.4	98	1.6
	LOQ	3	0.005	0.005	96	1.1	91	2.4
	Blank	1	0.000	0.000	-	-	-	-
Nectar	200x LOQ	3	1.018	1.023	99	1.2	82	2.7
	LOQ	3	0.005	0.005	105	2.8	82	10.6
	Blank	1	0.000	0.000	-	-	-	-
Flowers	High	5	50.07	50.31	87	4.9	96	3.9
	LOQ	3	1.018	1.023	87	4.9	92	5.1
	200x LOQ	3	0.005	0.005	108	7.9	96	8.8
	Blank	1	0.000	0.000	-	-	-	-
Pollen	200x LOQ	3	1.018	1.023	102	2.6	99	1.0
	LOQ	3	0.005	0.005	106	1.2	76	1.3

	Blank	1	0.000	0.000	-	-	-	-
Bees	200x LOQ	3	1.018	1.023	79	7.8	78	4.5
	LOQ	3	0.005	0.005	99	4.3	110	8.3
	Blank	1	0.000	0.000	-	-	-	-

n = replicates

Table A 221: Recovery results from method verification (qualifier)

Matrix	Validation Level	N	Fortification Level [mg/kg] (for Spray solution [mg/L])		Cymoxanil 199.10 → 111.0 (qualifier)		Zoxamide 336.00 →159.0 (qualifier)	
			Cymoxanil	Zoxamide	Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	High	5	767.71	771.46	102	1.8	105	9.2
	200x LOQ	3	1.075	1.080	103	1.0	102	2.8
	LOQ	3	0.005	0.005	95	3.2	89	3.0
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Nectar	200x LOQ	3	1.018	1.023	102	1.6	83	1.7
	LOQ	3	0.005	0.005	100	6.6	105	15.7
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Flowers	High	5	50.07	50.31	*	*	94	5.6
	LOQ	3	1.018	1.023	*	*	91	3.5
	200x LOQ	3	0.005	0.005	*	*	93	5.0
	Blank	1	0.000	0.000	*	*	n.d.	-
Pollen	200x LOQ	3	1.018	1.023	101	2.4	98	2.1
	LOQ	3	0.005	0.005	103	9.8	73	0.9
	Blank	1	0.000	0.000	n.d.	-	n.d.	-
Bees	200x LOQ	3	1.018	1.023	73	8.7	80	7.6
	LOQ	3	0.005	0.005	80	6.8	102	7.5
	Blank	1	0.000	0.000	n.d.	-	n.d.	-

n = replicates
* confirmatory transition could not be evaluated quantitatively due to matrix effects

Accuracyand precision / repeatability

The recovery values were in the range of 70-110% with relative standard deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

Linearity was demonstrated for solvent calibration and all matrix matched calibration curves (8 concentration levels) of cymoxanil in the range of 0.100 to 10.0 µg/L and in the range of 0.099 to 9.89 µg/L for zoxamide. This covers ranges from about 20% of the LOQ to about 200% above 200x LOQ, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was 0.005 mg/L (for spray solution, corresponding to 2.3 µg/L of cymoxanil and zoxamide in diluted samples) and 0.005 mg/kg (for nectar, flowers, pollen, bees) of cymoxanil and zoxamide (corresponding to 0.51 µg/L of cymoxanil and zoxamide in diluted samples and samples extracts).

Limit of detection

The limit of detection (LOD) was 0.001 mg/L (for spray solution) and 0.001 mg/kg (for nectar, flowers, pollen and bees) of cymoxanil and zoxamide.

Matrix effects

Matrix effects > 20% were observed for flowers, nectar, bees and pollen samples. For pollen and bee extracts, a strong suppression with up to approximately 50% was demonstrated. The nectar extracts show a strong enhancement partially with over 300%. The flowers extracts show strong enhancement at low concentration and a slightly suppression at high concentrations. Therefore, matrix-matched standard solutions were used for the solid commodities.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) and monitoring at two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times as cymoxanil and zoxamide, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Storage stability of sample extracts and solvent standards

Stability of sample extracts was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. Sample extracts were stable for a period of 11 days at 4-10°C.The maximum storage period of sample extracts in this analytical phase was 6 days

Storage stability of frozen samples

For each matrix, freezer storage stability was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. The analytical results between stored and fresh samples showed that the samples of spray solutions, nectar, bees, pollen and flowers are stable under frozen conditions for ≥ 1 year. The maximum storage period of specimens of study 19 48 BFB 0001 was 350 days at ≤-18°C

Table A 222: Storage stability

Matrix	Sample	Fortification level [mg/kg] (for spray solution [mg/L])	Cymoxanil		Zoxamide	
			Mean recovery [%]	RSD [%]	Mean recovery [%]	RSD [%]
Spray solution	Stored	1.000	95	0.3	92	10.2
	Fresh	1.000	96	1.5	92	13.0
	Difference	-	1	-	0	-
Nectar	Stored	1.000	106	1.2	84	0.9
	Fresh	1.000	98	0.7	91	0.8
	Difference	-	8	-	7	-
Bees	Stored	1.000	87	1.4	72	1.5
	Fresh	1.000	97	0.6	78	1.6
	Difference	-	10	-	6	-
Pollen	Stored	1.000	80	0.7	106	13.9
	Fresh	1.000	98	2.1	105	1.0
	Difference	-	18	-	1	-
Flowers	Stored Fresh	1.000	98	11.9	96	16.7
		1.000	108	0.8	108	1.4

	Difference	-	10	-	12	-

Table A 223: Characteristics of the analytical method for spay solution

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c. Calibration curve equation: $y = 14885.5 x + 3451.72$, $r^2=0.99984$	8-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: $y = 22687.6 x + 8715.03$, $r^2=0.99986$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/L*, corresponding to 2.30 µg/L in the analytical sample. LOD = 0.001 mg/L*, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/L, corresponding to 2.31 µg/L in the analytical sample. LOD = 0.001 mg/L, corresponding to 0.099 µg/L in the analytical sample

Table A 224: Characteristics of the analytical method for nectar

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 6280.32 x + 2541.11$, $r^2=0.99927$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 37803.7 x + 32501.3$, $r^2=0.99945$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	detected (validation blank samples had no peaks >30% of the lowest validation samples).	detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 225: Characteristics of the analytical method for flowers

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 21132.7 x + 519.863$, $r^2=0.99853$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 42436.9 x + 9228.50$, $r^2=0.99972$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects is presented	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 226: Characteristics of the analytical method for pollen

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 31441.2 x + 252.450$, $r^2=0.99979$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 47678.9 x + 4721.60$, $r^2=0.99997$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.458 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.460 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

Table A 227: Characteristics of the analytical method for bees

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 30101.0 x – 509.377$, $r^2=0.99987$	8-point calibration with external standard/ matrix matched calibration (10% v/v matrix) The calibration was linear and weighed 1/c Calibration curve equation: $y = 16274.3 x + 366.132$, $r^2=0.99991$
Calibration range	0.100 to 10.0 µg/L in analytical samples	0.099 to 9.89 µg/L in analytical samples
Assessment of matrix effects	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no	Yes, see Biochem project No.: 18 35 CRB 0040. Recoveries of validation samples within 70-110%. No interfering peaks were detected (validation blank samples had no

	Cymoxanil	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
	peaks >30% of the lowest validation samples).	peaks >30% of the lowest validation samples).
Limit of determination/ quantification	LOQ = 0.005 mg/kg, corresponding to 0.509 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.100 µg/L in the analytical sample.	LOQ = 0.005 mg/kg, corresponding to 0.512 µg/L in the analytical sample. LOD = 0.001 mg/kg, corresponding to 0.099 µg/L in the analytical sample.

The following figures show a typical chromatogram.

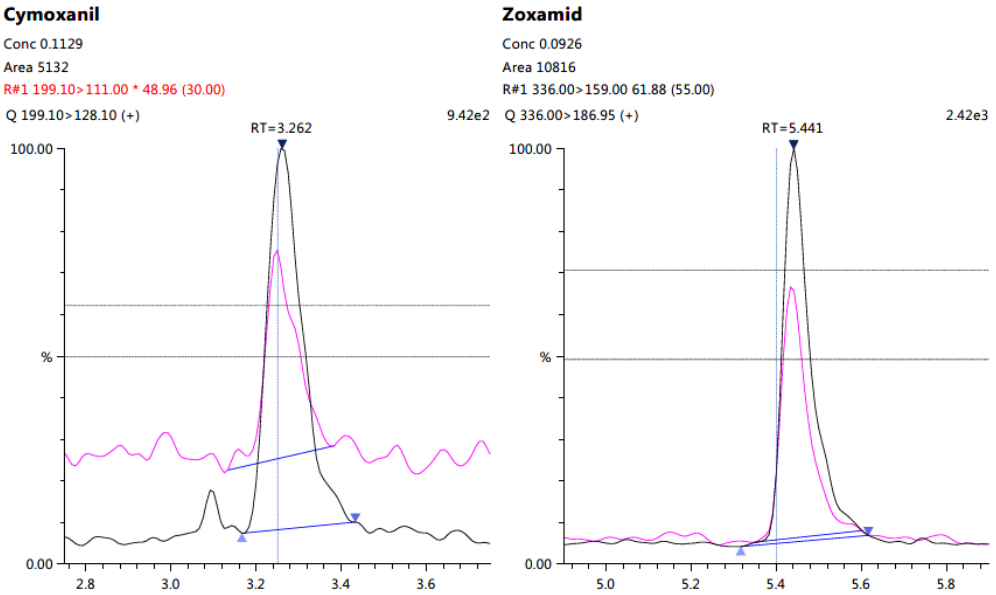


Figure A 93: Chromatogram of the lowest calibration standard (0.099 µg/L of cymoxanil (199.10 → 128.10 (quantifier)); 0.100 µg/L of zoxamide 336.00 → 186.95 (quantifier))

Conclusion

The method was verified on the test matrices spray solution, nectar, bees, pollen and flowers spiked with the test item (Cymoxanil 33% + Zoxamide 33% WG) at LOQ (0.005 mg/kg) and 200x LOQ (1 mg a.i./kg). For spray solutions and flowers, an additional validation level was analysed in order to cover the residues found in the original specimens. As a result, all requirements of SANCO/3029/99 rev. 4 and SANCO/825/00 rev 8.1 were fulfilled. For method validation details, please refer to the analytical phase report of BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001

(Schnurr A. 2020)

A 2.2.1.4 Description of analytical methods for the determination of residues in wine

and table grapes

A 2.2.1.4.1 Analytical method 1

A 2.2.1.4.1.1 Method validation

Comments of zRMS:	Studies 5.1/14,15 were already provided and assessed during the product authorisation.

Reference:	**KCA 5.1/14**
Report:	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) Following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Northern Europe 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2117, GLP, Not published
Guideline(s):	SANCO/825/00 rev.7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviation(s):	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCA 5.1/15**
Report:	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity grape (bunch, must young and bottled wine) following five applications of HARPON WG (Cymoxanil 33% + Zoxamide 33% WG) - Four trials, Northern Europe 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2120, GLP, Not published
Guideline(s):	SANCO/825/00 rev.7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviation(s):	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

A sample of bunches was taken and analyzed straight after deep freezing. For Cymoxanil the sample was extracted by Ultra-Turrax (bunch samples) or by shaking (processed samples) with ethyl acetate, purified by liquid-liquid partition and analysed by gas chromatography with a Nitrogen Phosphorus Detector (GC/NPD). Zoxamide was determined using an internal analytical method that consisted of a solvent extraction by Ultraturrax (bunch samples) or by shaking (processed samples). The extract containing the active ingredient was cleaned up by liquid-liquid partition and by SPE chromatography, then analysed by gas

chromatography equipped with an ECD. The Limit of Quantification (LOQ) was 0.05 mg/kg for cymoxanil on bunches samples and 0.01 mg/kg on processed samples; for zoxamide the LOQ was 0.01 mg/kg for all matrices. Limit of Detection (LOD) was 0.0290 mg/kg for bunch samples and 0.0058 mg/kg for processed samples (must, young wine and bottled wine) for cymoxanil, and 0.005 mg/kg for zoxamide, all matrices.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.25175 µg/mL to 2.51750 µg/mL (cymoxanyl) and 0.01019 µg/mL to 0.1019 µg/mL (zoxamide). The linear correlation was calculated for each analyte in wine matrix:

- cymoxanil
  r2 =0.999939 Y= 0.025487X+0.061535 (n= 6)
- Zoxamide
  r2 = 0.99006 Y= 0.000011X+0.006646 (n= 6)

Recoveries of zoxamide

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR01/01C/1R	Grapes / Bunch	0.01	74.58
I/CZ10/GR01/01C/2R	Grapes / Bunch	0.1	71.93
I/CZ10/GR01/01C/4R	Grapes / Bunch	0.5	70.70
I/CZ10/GR01/01C/5R	Grapes / Bunch	0.5	86.87
I/CZ10/GR01/01C/6R	Grapes / Bunch	0.5	85.22
I/CZ10/GR04/09C/1R	Grapes / Bunch	0.01	72.62
I/CZ10/GR04/09C/2R	Grapes / Bunch	0.1	77.82
Overall mean recovery			**77.11**
Overall standard deviation			**6.53**
Overall relative standard deviation (RSD)			**8.5**

Table 2: Recovery test result on grapes / bunch samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR02/05C/MU/1R	Must	0.01	86.36
I/CZ10/GR02/05C/MU/2R	Must	0.1	71.54
I/CZ10/GR02/05C/YW/1R	Young Wine	0.01	73.60
I/CZ10/GR02/05C/YW/2R	Young Wine	0.1	95.19
I/CZ10/GR02/05C/BW/1R	Bottled Wine	0.01	79.49
I/CZ10/GR02/05C/BW/2R	Bottled Wine	0.1	73.31
Overall mean recovery			**79.92**
Overall standard deviation			**9.26**
Overall relative standard deviation (RSD)			**11.6**

Table 3: Recovery test result on grapes / processed samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR01/01C/1R	Grapes / Bunch	0.01	74.58
I/CZ10/GR04/09C/1R	Grapes / Bunch	0.01	72.62
Overall mean recovery			**73.60**
Overall standard deviation			**1.39**
Overall relative standard deviation (RSD)			**1.9**

Table 4: Recovery test result on grapes / bunch samples at LOQ (0.01 mg/kg) spiking level

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/GR02/05C/MU/1R	Must	0.01	88.36
I/CZ10/GR02/05C/YW/1R	Young wine	0.01	73.60
I/CZ10/GR02/05C/BW/1R	Bottled wine	0.01	79.49
Overall mean recovery			79.82
Overall standard deviation			6.39
Overall relative standard deviation (RSD)			8.0

Table 5: Recovery test result on grapes / processed samples at LOQ (0.01 mg/kg) spiking level

Recoveries of cymoxanil

Analytical code	matrix	Cymoxanil	
		Level added (mg/kg)	Recovery %
G/CZ10/GR01/01C/1R	Grapes Bunch	0.05	95.73
G/CZ10/GR01/01C/2R	Grapes Bunch	0.5	87.96
G/CZ10/GR01/01C/3R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/4R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/5R	Grapes Bunch	*N.A.*	*N.A.*
G/CZ10/GR01/01C/6R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR03/07C/1R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR03/07C/2R	Grapes Bunch	*N.A.*	*N.A.*
F/CZ10/GR04/09C/1R	Grapes Bunch	0.05	95.13
F/CZ10/GR04/09C/2R	Grapes Bunch	0.5	84.31
Overall mean recovery			**90.78**
Overall standard deviation			**5.57**
Overall relative standard deviation (RSD)			**6.1**

(1)*N.A.: not applicable*

Analytical code	matrix	Cymoxanil	
		Level added (mg/kg)	Recovery %
F/CZ10/GR02/05C/MU/1R	Must	0.01	106.26
F/CZ10/GR02/05C/MU/2R	Must	0.1	106.95
Overall mean recovery			**106.61**
Overall standard deviation			**0.49**
Overall relative standard deviation (RSD)			**0.5**
F/CZ10/GR02/05C/YW/1R	Young Wine	0.01	99.30
F/CZ10/GR02/05C/YW/2R	Young Wine	0.1	94.04
Overall mean recovery			**96.67**
Overall standard deviation			**3.72**
Overall relative standard deviation (RSD)			**3.8**
F/CZ10/GR02/05C/BW/1R	Bottled Wine	0.01	101.47
F/CZ10/GR02/05C/BW/2R	Bottled Wine	0.1	105.97
Overall mean recovery			**103.72**
Overall standard deviation			**3.18**
Overall relative standard deviation (RSD)			**3.1**

(Romanini M. 2011)

A 2.2.1.5 Description of analytical methods for the determination of residues in tomato

A 2.2.1.5.1 Analytical method 1

A 2.2.1.5.1.1 Method validation

Comments of zRMS:	This method is already accepted in zoxamide section.

Reference:	**KCP 5.1/18**
Report	Tetuan, B., 2016: Determination of residues at harvest of zoxamide, benalaxyl-m and cymoxanil in tomato, following three broadcast applications of GWN-10392, GWN-9823 and IR6141-copper oxychloride-copper hydroxide 5-15-15 WG under greenhouse conditions and determination of residues at harvest of zoxamide and benalaxyl-m in industry tomato and its processed products (canned tomatoes, puree and juice), following three broadcast applications of GWN-10392, under open field conditions - South Europe - season 2015 Gowan Comercio Internacional e Servicos Limitada, Portugal Promovert Crop Services SL, Spain, Report No. 15 F CL GW P/A, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) SANCO/3029/99 rev. 4 (2000)
Deviations:	Deviation to analytical lab SOP: the weighing of benalaxyl-m for the preparation of the 1 g/L stock solution was less than 10 mg (9.82 mg). This deviation was regarded to have no impact on the results of the study since the balance allows to weigh less 2 mg. Deviation to the study plan: analyses and an analytical phase-based inspection were performed before the finalisation of a study plan amendment – there was no impact on the results as the first daily sample set was not validated. However, the analytical phase-based inspection performed on the 31st December 2015 was kept as the analytical method was the same for the following samples sets.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Residues of benalaxyl-m, cymoxanil and zoxamide in tomato fruits and processed commodities (canned tomatoes, tomato puree and tomato juice) were analysed at Fredon Pays de la Loire / GIRPA in France under report no. B15G-P2-BCZ-01 with a method validated according to SANCO/3029/99 rev. 4 under report no. B15G-P2-BCZ-01. The analytical method principle was based on the method European Committee for Standardisation (CEN): EN 15662:2009-02. "Foods of plant origin – Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE – QuEChERS-method" using HPLC-MS/MS.

Accurately weigh 10 g of ground laboratory sample into a 50 mL polypropylene centrifuge tube. Extract before defrosting. For recoveries, fortify the sample with the appropriate spiking standards solutions using a pipette. Using a measuring cylinder, add about 10 mL of acetonitrile (HPLC quality). Shake manually and vigorously during 1-minute. Transfer the crude extract into a 50 mL QuEChERS tube containing a $MgSO_4$/NaCl/salt tampon. Immediately shake manually and vigorously for 1 minute. Centrifuge for 5 minutes at 4000 rpm. Use an automatic pipette to fill a 6 mL of the organic phase into a 15 mL QuEChERS

tube containing 900 mg of $MgSO_4$ and 150 mg of PSA. Shake manually and vigorously during 1 minute. Centrifuge for 5 minutes at 4000 rpm. Dilute twice the clear extract into ultra-pure water. Two mass transitions were monitored for benalaxyl-m, cymoxanil and zoxamide. The limit of quantification (LOQ) was set at 0.01 mg/kg per analyte.

The analytical method used was validated on tomato fruits within this analytical phase by 10 spiked samples, 5 recovery experiments fortified at the LOQ level, 5 recovery experiments fortified at ten times the LOQ level, 2 control samples and a reagent blank.

Equipment

Instrument: 5500 QTRAP, Autosampler Exsigent, PAL HTC-xt, Pump LC Shimadzu LC-20AD XR

Column: C_{18} Hydro RP (100 mm x 3 mm ID x 2.5 µm PD)

Mobile phase: A: Ultra-pure water / glacial acetic acid (100/0.1) (v/v) + 5 mM ammonium acetate
B: Methanol / glacial acetic 100: 0.1 (v/v) + 5 mM ammonium acetate

Time table (min)	A (%)	B (%)
0.0	100	0
0.1	100	0
4.0	0	100
6.4	0	100
6.5	100	0
8.0	100	0

Flow rate: 0.7 mL/min

Column temp.: 60°C

Injection volume: 20 µL

Retention time: ~ 4.9 min for benalaxyl-M
~ 3.5 min for cymoxanil
~ 4.8 min for zoxamide

Ionisation: MRM (Positive Multiple reaction Monitoring)

Ion mode
Benalaxyl-m: m/z 326 to m/z 148 (quantification)
m/z 326 to m/z 208 (qualification)
Cymoxanil: m/z 199 to m/z 128 (quantification)
m/z 199 to m/z 111 (qualification)
Zoxamide: m/z 336 to m/z 187 (quantification)
m/z 336 to m/z 159 (qualification)

For mass spectrometer conditions, please refer to the study report.

Results and discussions

Table A 228: Recovery results of benalaxyl-m, cymoxanil and zoxamide

Specimen	Reference items	Level spiked (mg/kg)	Mean recovery rate (%)	RSD (%)	Number of recovery rates (n)
Tomato fruits	Benalaxyl-m	0.010	109	4	5
		0.100	109	8	5
		Overall	109	6	10

Specimen	Reference items	Level spiked (mg/kg)	Mean recovery rate (%)	RSD (%)	Number of recovery rates (n)
	Cymoxanil	0.010	104	4	5
		0.100	106	6	5
		Overall	105	5	10
	Zoxamide	0.010	104	5	5
		0.100	104	9	5
		Overall	104	7	10

Accuracy and repeatability/precision

Mean recoveries per fortification level were around 100 % with % RSD values less than 20%, the method therefore fulfils the requirements for residue analytical methods. Thus, demonstrating a satisfying accuracy and precision of the method.

Linearity

Linearity of the calibration curve was demonstrated between 1.5 to 20 µg/L for benalaxyl-m and zoxamide and 1.5 to 200 µg/L for cymoxanil over at least 5 measurement points. The correlation coefficients were typically higher than 0.99.
The analytical calibration spans over a range including the lowest and highest nominal concentration of the reference item in the analytical solutions ± at least 20%.

Limit of quantification (LOQ)

The limit of quantification of the method was set at the lowest validated level where a mean recovery within the range 70-110% with a RSD less or equal to 20% could be obtained. The LOQ for benalaxyl-m, cymoxanil, zoxamide is 0.01 mg/kg.

Matrix effects

Any matrix effects were compensated using matrix-matched standard. Analysis of control specimens of tomato fruits yielded no residues of benalaxyl-m, cymoxanil or zoxamide above 30% of the limit of quantification, indicating that no interference was present at the retention time of these reference items.

Specificity

The specificity was checked by analysis of at least one untreated specimen (two repetitions) and at least one reagent blank. Interferences due the substrate were less than 30% of the limit of quantification.

The method using LC-MS/MS is regarded as specific, monitoring two ion mass transitions.

Stability of sample extracts

Final sample extracts were analysed within 24 hours.

Storage stability of frozen samples

The maximal storage interval between sampling and analysis was 153 days.

Table A 229: Characteristics for the analytical method validation for the determination of benalaxyl-m, cymoxanil and zoxamide residues in tomatoes

	Benalaxyl-m	**Cymoxanil**	**Zoxamide**
Specificity	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ	LC-MS/MS method monitoring two ion transitions. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standardcalibration, 5 points calibration; r^2>0.99; linear Regression Model y = 1003528,51x + 186828,04 Individual calibration data and calibration line equation presented in the study report	Matrix matched standardcalibration, 8 point calibration; r^2>0.99; linear. Regression Model y = 66329,88x + 1929,67 Individual calibration data and calibration line equation presented in the study report	Matrix matched standardcalibration, 5 points calibration; r^2>0.99; linear Regression Model y = 291380,50x + 45430,50 Individual calibration data and calibration line equation presented in the study report
Calibration range	1.5 to 20 µg/L^{-1}	1.5 to 200 µg/L^{-1}	1.5 to 20 µg/L^{-1}
Assessment of matrix effects is presented	Yes	Yes	yes
Limit of quantification (LOQ)	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg

The following figures show chromatograms

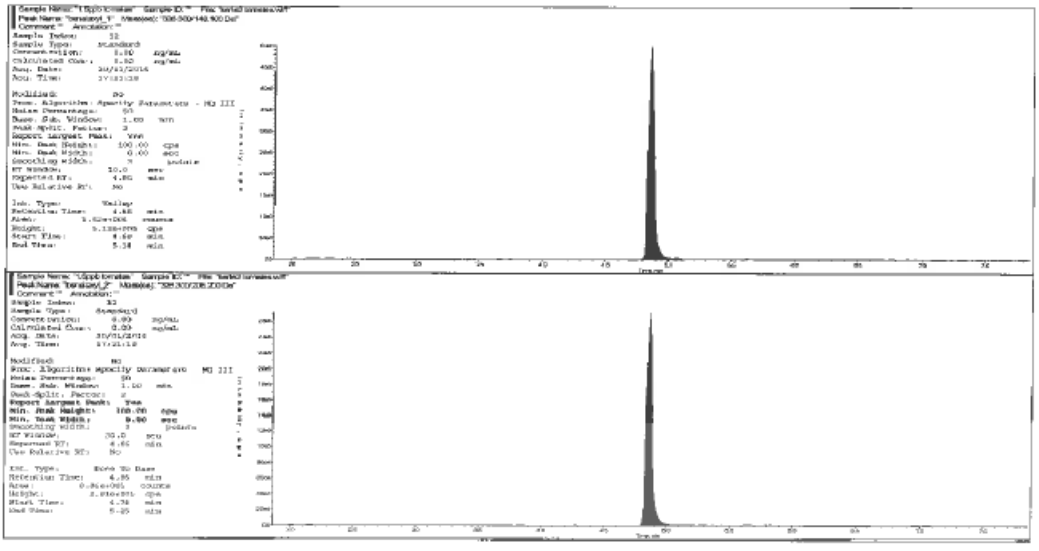


Figure A 94: Typical chromatogram of benalaxyl-m

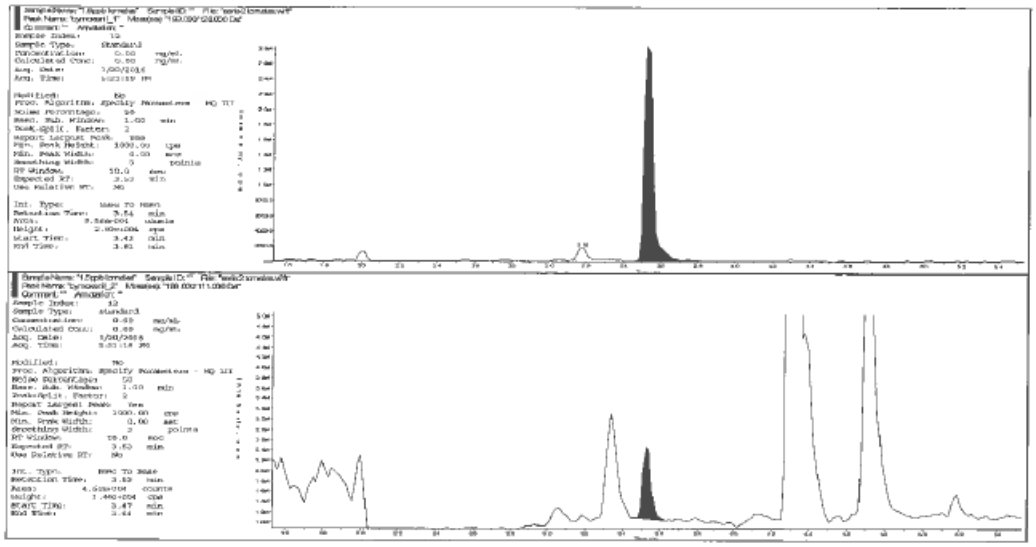


Figure A 95: Typical chromatogram of cymoxanil

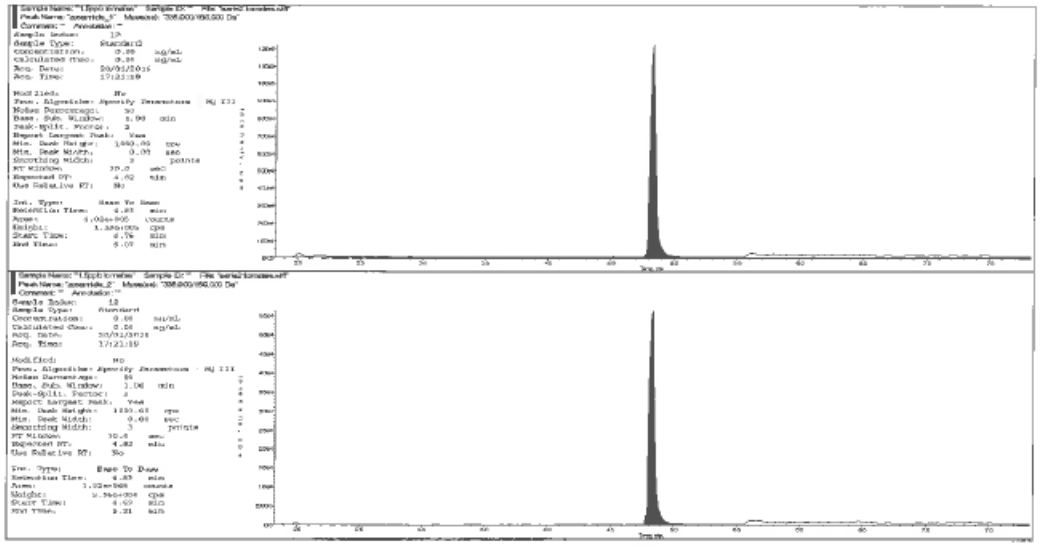


Figure A 96: Typical chromatogram of zoxamide

Conclusion

An LC-MS/MS method (QuEChERS-method) for the determination of zoxamide, cymoxanil and benalaxyl-m in tomato fruits and processed commodities (canned tomatoes, tomato puree and tomato juice) has been successfully validated according to SANCO/3029/99 rev. 4 with an LOQ of 0.01 mg/kg.

(Tetuan B. 2016)

A 2.2.1.5.2 Analytical method 2

A 2.2.1.5.2.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/19**
Report	Romanini, M., 2011: Determination of cymoxanil and zoxamide residues at harvest in raw and processed agricultural commodity tomato (fruit, juice, puree and canned) following five applications of HARPON WG (Cymoxanil 33% + zoxamide 33% WG) - Four trials, Italy 2010 Gowan Comercio Internacional e Servicos Limitada, Portugal Research Centre "E. Gagliardini", Italy, Report No. CREG2118, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000) SANC0/825/00 rev.7 (2004)
Deviations:	No
Acceptability:	Yes

Materials and methods

For cymoxanil the sample was extracted by Ultra-Turrax with ethyl acetate, purified by liquid-liquid partition and analysed by gas chromatography with a Nitrogen Phosphorus Detector (GC/NPD). Zoxamide was determined using an internal analytical method that consisted in a solvent extraction by Ultraturrax (bunch samples) or by shaking (processed samples). The extract containing the active ingredient was cleaned up by liquid-liquid partition and by SPE chromatography, then analysed by gas chromatography equipped with an ECD. The Limit of Quantification (LOQ) was 0.05 mg/kg for cymoxanil on tomato samples and 0.01 mg/kg on processed samples; for zoxamide the LOQ was 0.01 mg/kg for all matrices. Limit of Detection (LOD) was 0.0199 mg/kg for tomato samples and 0.0249 mg/kg for processed samples (juice, puree and canned) for cymoxanil, and 0.0051 for zoxamide in all matrices.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.4980 µg/mL to 2.490 µg/mL (cymoxanil) and 0.005130 µg/mL to 0.1028 µg/mL (zoxamide). The linear correlation was calculated for each analyte in wine matrix:

- Cymoxanil
  r2 =0.999909 Y= 0.081470X+0.089616 (n= 6)
- Zoxamide
  r2 = 0.991140 Y= 0.000011X+0.000929 (n= 6)

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO01/01C/1R	Tomato / Fruit	0.01	97.76
I/CZ10/TO01/01C/2R	Tomato / Fruit	0.1	94.55
I/CZ10/TO02/03C/1R	Tomato / Fruit	0.01	105.99
I/CZ10/TO02/03C/2R	Tomato / Fruit	0.1	100.16
I/CZ10/TO03/07C/2R	Tomato / Fruit	0.1	109.55
I/CZ10/TO03/07C/3R	Tomato / Fruit	0.01	107.21
I/CZ10/TO03/07C/4R	Tomato / Fruit	0.01	102.34
I/CZ10/TO04/09C/1R	Tomato / Fruit	0.01	108.19
I/CZ10/TO04/09C/2R	Tomato / Fruit	0.1	97.17
I/CZ10/TO04/09C/3R	Tomato / Fruit	0.5	84.68
I/CZ10/TO04/09C/4R	Tomato / Fruit	0.5	81.52
Overall mean recovery			**99.01**
Overall standard deviation			**9.27**
Overall relative standard deviation (RSD)			**9.4**

Table 2: Recovery test result on tomato / fruit samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO02/05C/JU/1R	Tomato / Juice	0.01	97.47
I/CZ10/TO02/05C/JU/2R	Tomato / Juice	0.1	86.55
I/CZ10/TO02/05C/PU/1R	Tomato / Puree	0.01	81.87
I/CZ10/TO02/05C/PU/2R	Tomato / Pureee	0.1	93.66
I/CZ10/TO02/05C/CA/1R	Tomato / Canned	0.01	108.19
I/CZ10/TO02/05C/CA/2R	Tomato / Canned	0.1	93.08
Overall mean recovery			**93.47**
Overall standard deviation			**9.11**
Overall relative standard deviation (RSD)			**9.8**

Table 3: Recovery test result on tomato / processed samples

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO01/01C/1R	Tomato / Fruit	0.01	97.76
I/CZ10/TO02/03C/1R	Tomato / Fruit	0.01	105.99
I/CZ10/TO03/07C/3R	Tomato / Fruit	0.01	107.21
I/CZ10/TO03/07C/4R	Tomato / Fruit	0.01	102.34
I/CZ10/TO04/09C/1R	Tomato / Fruit	0.01	108.19
Overall mean recovery			**104.30**
Overall standard deviation			**4.27**
Overall relative standard deviation (RSD)			**4.1**

Table 4: Recovery test result on tomato / fruit samples at LOQ (0.01 mg/kg) spiking level

Analytical code	matrix	Zoxamide	
		Spiking level (mg/kg)	Recovery %
I/CZ10/TO02/05C/JU/1R	Tomato / Juice	0.01	97.47
I/CZ10/TO02/05C/PU/1R	Tomato / Puree	0.01	81.87
I/CZ10/TO02/05C/CA/1R	Tomato / Canned	0.01	108.19
Overall mean recovery			**95.84**
Overall standard deviation			**13.24**
Overall relative standard deviation (RSD)			**13.8**

Table 5: Recovery test result on tomato / processed samples at LOQ (0.01 mg/kg) spiking level

(Romanini M. 2011)

A 2.2.1.6 Description of analytical methods for the determination of residues in potatoes

A 2.2.1.6.1 Analytical method 1

A 2.2.1.6.1.1 Method validation

Comments of zRMS:	Studies 5.1/21, 22 was already provided and assessed during the product authorisation.

Reference:	**KCP 5.1/21**
Report	Tetuan, B., 2011: Determination of residues at harvest in potatoes, following six broadcast applications of Harpon WG, under field conditions - Northern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/A, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	**KCP 5.1/22**
Report	Tetuan, B., 2011: Determination of residues at harvest in potatoes, following five broadcast applications of HARPON WG, under field conditions - Southern Europe - season 2010 Gowan Comercio Internacional & Servicos Ltda, Portugal PROMO-VERT, France, Report No. 10 F PT GW P/B, PROMO/ZOX-CYM/10.01, GLP, Not published
Guideline(s):	SANCO/825/00 rev. 7 (2004) SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Residue concentrations were determined by LC/MS/MS analysis using method based on **GIRPA (GIR/MET/CYMOXANI/04V1 and GIR/MET/ZOXAMIDE/02V1 Analytical method was based on the multiresidue QuEChERS method (Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - European method NF EN 15662) and on the Dow AgroSciences GRM 02.07.R1 method (Residue analysis of**

zoxamide in grapes, potatoes and tomatoes using GC-MS detection – 09/12/2002 – Ana Cristina Pinheiro and Roberto De Vito).

Residues of cymoxanil and zoxamide are extracted from potatoes in frozen condition with the help of an acetonitrile/2% potassium bicarbonate aqueous solution (80/20, v/v) mixture. After addition of magnesium sulfate, sodium chloride and buffering citrate salts, the mixture was shaken intensively and centrifuged for phase separation. An aliquot of the organic phase was cleaned-up by dispersive solid phase extraction (D-SPE). **For each reference item,** the determination was performed by liquid chromatography with detection by mass spectrometric in tandem (LC-MS/MS) **with two different mass transitions, one for quantification and one for qualification.**

Results

Specificity

For each reference item, the specificity of the method has been demonstrated; interferences due to the substrate were less than 30% of the limit of quantification.
The reagent blank showed that no interference due to the reagents was detected.
The chromatographic method on LC-MS/MS **following two transitions** is highly specific, an additional confirmatory method was not necessary.

Qualitative confirmation was carried out by comparison of the relative abundances of the qualification transition (% relative to quantification transition) in spiked sample extracts, with those in the calibration standards.
Summary of quantification/qualification transitions used:

Summary of quantification/qualification transitions used:

Reference item	*Quantification transition*	*Qualification transition*
Cymoxanil	*199 → 128*	*199 → 111*
Zoxamide	*336 → 187*	*336 → 159*

All the transitions are specific for each reference item. The most sensitive transitions were chosen for quantification.

Linearity

For cymoxanil and zoxamide the linearity was checked on a range of concentration from 0.004 mg/L to 0.030 mg/L. The linear correlation was calculated for each analyte in potato matrix:

- Cymoxanil
  r2 =0.999966 Y= 10452X+1325.7 (n= 5)
- Zoxamide
  r2 = 1 Y= 34910X+11437 (n= 5)

LOQ = 0.01 mg/kg for both substances.

Repeatability/Accuracy

Specimen	Reference item	Level spiked ($mg.kg^{-1}$)	Recovery rate (%)	Relative standard deviation (%)	Number of recovery rates (n)
Potatoes	Cymoxanil	0.010	101	4	5
		0.100	110	5	5
		all	106	6	10
	Zoxamide	0.010	104	2	5
		0.100	110	5	5
		all	107	5	10

(Tetuan B. 2011)

A 2.2.1.7 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

Additional methods validation studies for cymoxanil have been provided below, since these methods were used in storage stability studies for tomatoes and grapes. Study A0087 presented in cymoxanil DAR Vol. 3, B.5 represents the ILV study for these methods.

A 2.2.1.7.1 Analytical method 1

A 2.2.1.7.1.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.2/05**
Report	Freschi, G., 2001: Validation of analytical method for determination of residues of cymoxanil in tomato (whole fruit) Oxon Italia S.p.A., Italy Sipcam S.p.A, Italy, Report No. SIP1278, GLP, Not Published
Guideline(s):	SANCO 3029/99
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary

Materials and methods

Cymoxanil was extracted from samples with 150 ml ethylacetate, using ultra turrax at 13500 rpm for five minutes. Anhydrous sodium sulphate was added, the mixture was filtered and rinsed for three times with 50 mL of Ethylacetate.

The filtrate was collected and evaporated; 50 ml of distilled water was added and the aqueous extract was washed with 30 mL of hexane and then extracted twice with 50 mL of dichloromethane.

A purification step was carried out on silica gel column: 10g of silica gel in 20 mL of n-hexane and transferred in the column and anhydrous sodium sulphate was added.

The column was eluted with 200 mL of a mixture of Ethylacetate/n hexane (10/90 v/v) and this fraction was discarded. Cymoxanil was eluted with 350 mL of mixture ethylacetate/n-hexane (50/50 v/v). The solvent was evaporated and the dry extract dissolved in a small amount of acetone (5 mL).

Finally the purified extract was analysed by gas chromatography equipped with a nitrogen specific detector (NPD).

Equipment

GC/NPD:	Helwlett Packard mod. 5890 equipped with a NPD detector
Column:	DB-5 in fused silica, 30m x 0.53 mm,. 1.5µm film thickness
Injection temp.:	250°C
Injection volume:	3µL
Retention time:	~11 min

Results and discussions

Specificity

The retention time of the analyte into the samples was compared with the retention time of standard solutions showing a good match.
Additionally no interferences were noted in the blank samples and in the processed blank samples they didn't exceed the 30% of LOD.

Linearity

The linearity of the response was shown to be linear in the range 0.5506 – 2.7531µg/mL.
Cymoxanil calibration curve: y = 0.02818253 x + 0.163288 (r > 0.989).

Accuracy

Samples were fortified at 500µg/kg and 50 µg/kg, 5 recoveries for each fortification level were performed.
A mean recovery of 96.1%, with a standard deviation of 3.3% and a C.V of 3.4% were calculated for the higher fortification level.
For the lower fortification level a mean recovery of 103.7%, with a standard deviation of 11.7% and a C.V of 10.9% were calculated
Overall recovery was calculated to be 99.9%, with a standard deviation of 8.8% and a C.V. of 8.8%.

Limit of quantification

The LOQ of the method was defined as the lowest fortification level at which acceptable recovery data was obtained. A LOQ of 0.05 mg/kg was therefore calculated.

Limit of detection

The LOD was defined as the concentration of the lowest calibration standard chromatographed. It was calculated to be 0.02 mg/kg.

Table A 230: Characteristics for the analytical method used for validation of cymoxanil residues in plant matrices

	Cymoxanil
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration. Calibration range 0.5506 – 2.7531µg/mL linear. Regression Model y = 0.02818253 x + 0.163288. Correlation coefficient r^2 was >0.989. Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.5506 – 2.7531µg/mL
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.05 mg/kg

(Freschi G. 2001)

A 2.2.1.7.2 Analytical method 2

A 2.2.1.7.2.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.2/06**
Report	Freschi, G., 2001: Validation of analytical method for determination of residues of cymoxanil in grapes (bunches) Oxon Italia S.p.A., Italy Sipcam S.p.A, Italy, Report No. SIP1276, GLP, Not Published
Guideline(s):	SANCO 3029/99
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Cymoxanil was extracted from grapes with 150 ml ethylacetate, using ultra turrax at 13500 rpm for five minutes. Anhydrous sodium sulphate was added; the mixture was filtered and rinsed for three times with 50 mL of Ethylacetate.

The filtrate was collected and evaporated; 50 ml of distilled water was added and the aqueous extract was washed with 30 mL of hexane and then extracted twice with 50 mL of dichloromethane.

A purification step was carried out on silica gel column: 10g of silica gel in 20 mL of n-hexane and transferred in the column and anhydrous sodium sulphate was added.

The column was eluted with 200 mL of a mixture of Ethylacetate/n hexane (10/90 v/v) and this fraction was discarded. Cymoxanil was eluted with 350 mL of mixture ethylacetate/n-hexane (50/50 v/v). The solvent was evaporated and the dry extract dissolved in a small amount of acetone (5 mL).

Finally the purified extract was analysed by gas chromatography equipped with a nitrogen specific detector (NPD).

Equipment

GC/NPD:	Helwlett Packard mod. 5890 equipped with a NPD detector
Column:	DB-5 in fused silica, 30m x 0.53 mm,. 1.5µm film thickness
Injection temp.:	250°C
Injection volume:	3µL
Retention time:	~11 min

Results and discussions

Specificity

The retention time of the analyte into the samples was compared with the retention time of standard solutions showing a good match.
Additionally no interferences were noted in the blank samples and in the processed blank samples they didn’t exceed the 30% of LOD.

Linearity

The linearity of the response was shown to be linear in the range 0.5097 – 2.5487µg/mL.
Cymoxanil calibration curve: y = 0.01917 x + 0.103308 (r > 0.999).

Accuracy

Samples were fortified at 500µg/kg and 50 µg/kg, 5 recoveries for each fortification level were performed.
A mean recovery of 97.9%, with a standard deviation of 8.5% and a C.V of 8.7% were calculated for the higher fortification level.
For the lower fortification level a mean recovery of 97.0%, with a standard deviation of 5.6% and a C.V of 5.8% were calculated
Overall recovery was calculated to be 97.4%, with a standard deviation of 6.8% and a C.V. of 7.0%.

Limit of quantification

The LOQ of the method was defined as the lowest fortification level at which acceptable recovery data was obtained. A LOQ of 0.05 mg/kg was therefore calculated.

Limit of detection

The LOD was defined as the concentration of the lowest calibration standard chromatographed. It was calculated to be 0.02 mg/kg.

Table A 231: Characteristics for the analytical method used for validation of cymoxanil residues in plant matrices

	Cymoxanil
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration. Calibration range 0.5506 – 2.7531µg/mL linear. Regression Model y = 0.02818253 x + 0.163288. Correlation coefficient r^2 was >0.989. Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.5506 – 2.7531µg/mL
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.05 mg/kg

(Freschi G. 2001)

A 2.2.1.8 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new data.

A 2.2.1.9 Description of Methods for the Analysis of Soil (KCP 5.2)

No new data.

A 2.2.1.10 Description of Methods for the Analysis of Water (KCP 5.2)

A 2.2.1.10.1 Analytical method 1

A 2.2.1.10.1.1 Method validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.2/07**
Report	Leak, T., 2010: Analytical method for the determination of Cymoxanil and IN-KQ960 in water (pond, stream, well and tap) using LC/MS/MS Oxon Italia S.p.A., Italy ChemService, Italy, Report No. DuPont-27500/ ABC-65072 rev.2, GLP, Not Published
Guideline(s):	SANCO/825/00 rev.7
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary

Materials and methods

The analytical method for the determination of cymoxanil and its metabolite IN-KQ960 in water (pond, stream, well and tap) was validated at 0.100 and 1.00 ppb in all matrices using an LC/MS/MS system operating in turbo ion spray (TIS) in positive ion mode.

Water samples were prepared by placing 10 ml of the test sample into a culture tube, removing an aliquot of water equal to the volume of the intended fortification, and fortifying with appropriate spiking solution. The samples were then injected.

Equipment

HPLC System:	Agilent coupled to an Applied Biosystem API-4000 triple quadrupole mass spectrometer with a turbo ion spray interface (TIS)
Column:	Phenomenex Luna C8, 2 mm x 150 mm, 5 µm
Column temperature:	30°C
Injection volume:	40µL
Interface:	Turbo Ion Spray (TIS)
Retention time:	Cymoxanil: ~7.19 min IN-KQ960: ~ 4.57 min
Solvents:	A: 0.1% formic acis B HPLC- grade methanol
Transitions:	Cymoxanil: 199.1 → 128.1 IN-KQ960: 217.1 → 146.0

Results and discussions

Recovery Findings

Table A 232: Recovery results

Matrix		Cymoxanil	IN-KQ960

	Level (ng/mL)	Recovery ±RSD	Range	N	Recovery ±RSD	Range	N
Pond water	0.100	81 ± 5.30	76-85	5	93 ± 4.69	89-100	5
	1.00	79 ± 3.32	77-83	5	98 ± 2.22	96-101	5
Stream water	0.100	84 ± 3.77	79-87	5	98 ± 3.84	94-103	5
	1.00	85 ± 1.04	83-85	5	99 ± 1.41	98-101	5
Well water	0.100	96 ± 2.79	91-98	5	99 ± 6.42	91-105	5
	1.00	93 ± 3.20	89-96	5	104 ± 2.11	103-108	5
Tap water	0.100	92 ± 2.74	76-85	5	98 ± 6.10	89-100	5
	1.00	94 ± 0.811	93-95	5	103 ± 2.06	100-105	5

RDS: relative standard deviation
N: number of samples

Specificity

LC/MS/MS was used for instrumental analysis. No matrix interference was observed I the regions of cymoxanil and IN-KQ960 elution in chromatograms of control samples. For quantification transitions 199.1 → 128.1 and 218.1 → 146.0 were used for cymoxanil and IN-KQ960 respectively.

Linearity

For each analyte, calibration standards yielded a linear response (r^2 > 0.99) over the range of 0.300-2.00ng/mL.
Cymoxanil calibration curve: $y = 3.56e^{+5} x + 1.65e^{+3}$ (r = 0.9991)
IN-KQ960 calibration curve: $y = 9.42e^{+4} x + 415$ (r = 0.9987)

Accuracy

Water samples were fortified at 0.100 (LOQ) and 1.00 ppb (ng/mL). 5 recoveries for each matrix and each fortification level were performed.
Recoveries range from 79-96% for cymoxanil and 93-104% for IN-KQ960.
RSDs go from 0.811 to 5.30 for cymoxanil and from 1.41 to 6.42 for IN-KQ960.

Limit of quantification

The LOQ of the method for all matrices was 0.100 ppb (ng/mL). The MQL (0.0300 ng/mL) was calculated by multiplication of the low standard concentration times the analysis volume divided by the sample volume.

Table A 233: Characteristics for the analytical method used for validation of cymoxanil and IN-KQ960 residues in water

	Cymoxanil	IN-KQ960
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration Calibration range 0.300 to 2.0 ng/mL linear. Regression $y = 3.56e^{+5} x + 1.65e^{+3}$ (r = 0.9991) Correlation coefficient r^2 was ≥0.99 Individual calibration data and calibration line equation presented in the study report.	Standard solution and matrix matched calibration Calibration range 0.300 to 2.0 ng/mL linear. Regression $y = 9.42e^{+4} x + 415$ (r = 0.9987) Correlation coefficient r^2 was ≥0.99 Individual calibration data and calibration line equation presented in the study report.

	Cymoxanil	**IN-KQ960**
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration range	0.300 to 2.0 ng/mL	0.300 to 2.0 ng/mL
Assessment of matrix effects is presented	Yes	Yes
Limit of determination/quantification	0.1 ng/mL	0.1 ng/mL

Conclusion

In conclusion, the analytical method is suitable for the quantification of cymoxanil and IN-KQ960 in water matrices at an LOQ of 0.100 ppb.

(Leak T. 2010)

A 2.2.1.10.1.2 Independent laboratory validation

Comments of zRMS:	This study was already provided and assessed during the product authorisation.

Reference:	**KCP 5.2/08**
Report	Cermak, J. 2013: Independent Laboratory Validation for the Determination of residues of cymoxanil and IN-KQ960 in water (drinking and stream) using LC/MS/MS Oxon Italia S.p.A., Italy Research Institute for Organic Syntheses, Inc., Czech Republic, Report No. 2556-256/12/48 (DuPont-35792), GLP, Not published
Guideline(s):	SANCO/825/00 rev.8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary

Materials and methods

An independent laboratory validation was performed for the determination of residues of cymoxanil and IN KQ960 in drinking and stream water using an LC/MS/MS technique. The specimens were analysed monitoring two MRM transitions; the method was therefore highly specific and additional confirmatory method was not necessary (according to SANCO/825/00 rev. 8.1).

Water samples were prepared placing 9.9 mL of test sample into a culture tube and fortifying with appropriate spiking solution. Samples were homogenized by Vortex, vialed and analysed. Details of the analysis are reported below.

Equipment

HPLC System:	Agilent HP 1260 infinity HPLC coupled to an Agilent mass Hunter LC/MS/MS
MS System:	Agilent 6490 TripleQ MS
Column:	Nucleodur C8 Gravity EC150/2, 2 mm x 150 mm, 5 µm
Column temperature:	30°C

Injection volume:	90µL
Solvents:	A A: 0.1% formic acid + water
	B: methanol + 0.1% ammonium hydroxide
Retention time:	Cymoxanil: ~6.7 min
	IN-KQ960: ~ 4.1 min
Transitions:	Cymoxanil: 199 → 128 (quantification)
	199 → 111 (confirmation)
	IN-KQ960: 217 → 146 (quantification)
	217 → 71 (confirmation)

Results and discussions

Recovery Findings

Table A 234: Recovery results

Matrix	Level (mg/kg)	Cymoxanil recovery						IN KQ960 recovery					
		MRM 199 → 128 (quantification)			MRM 199 → 111 (confirmation)			MRM 217 → 146 (quantification)			MRM 217 → 71 (confirmation)		
		Mean (%)	Std. dev. (%)	RSD (%)	Mean (%)	Std. dev. (%)	RSD (%)	Mean (%)	Std. dev. (%)	RSD (%)	Mean (%)	Std. dev. (%)	RSD (%)
Drinking water	0.100	100	2.8	2.8	95	4.0	4.2	99	3.2	3.3	98	3.6	3.6
	1.00	101	5.7	5.7	99	5.5	5.5	96	6.1	6.4	95	5.8	6.1
Stream water	0.100	99	3.8	3.8	97	2.4	2.5	110	3.8	3.4	108	3.8	3.6
	1.00	96	1.5	1.6	94	1.7	1.8	108	1.5	1.4	108	1.7	1.7

RDS: relative standard deviation

Specificity

LC/MS/MS was used for instrumental analysis. No matrix interference was observed I the regions of Cymoxanil and IN-KQ960 elution in chromatograms of control samples. For quantification transitions 199 → 128 and 217 → 146.0 were used for Cymoxanil and IN-KQ960 respectively. Two additional transitions were also monitored 199 → 111 and 217 → 71 for cymoxanil and IN KQ960 respectively.

Linearity

The linearity of the response was confirmed by injecting seven standard solutions covering the range 0.03ng/mL – 2.0ng/mL. R^2 was found to be > 0.99 for all mass transitions.

Cymoxanil calibration curve: y = 243385 x + 1863.9 (r = 0.9999)
IN-KQ960 calibration curve: y = 39141 x + 220.09 (r = 0.9999)

Accuracy

Water samples were fortified at 0.100 (LOQ) and 1.00 ppb (ng/mL). 5 recoveries for each matrix and each fortification level were performed.
Recoveries range from 79-96% for cymoxanil and 93-104% for IN-KQ960.
RSDs go from 0.811 to 5.30 for cymoxanil and from 1.41 to 6.42 for IN-KQ960.

Limit of quantification

The LOQ of the method for all matrices was 0.100 ppb (ng/mL).

Limit of detection

The LOD (0.0300 ng/mL) was 30% of the LOQ. Chromatographic peaks were greater than the signal equivalent to three times the background noise.

Table A 235: Characteristics for the analytical method used for validation of cymoxanil and IN-KQ960 residues in water

	Cymoxanil	**IN-KQ960**
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Standard solution and matrix matched calibration Calibration range 0.03ng/mL to 2.0ng/mL linear. Regression y = 243385 x + 1863.9 (r = 0.9999). Correlation coefficient r^2 was ≥0.99 Individual calibration data and calibration line equation presented in the study report.	Standard solution and matrix matched calibration Calibration range 0.03 to 2.0 ng/mL linear. Regression y = 39141 x + 220.09 (r = 0.9999). Correlation coefficient r^2 was ≥0.99 Individual calibration data and calibration line equation presented in the study report.
Calibration range	0.03 to 2.0 ng/mL	0.03 to 2.0 ng/mL
Assessment of matrix effects is presented	yes	Yes
Limit of determination/quantification	0.1 ng/mL	0.1 ng/mL

Conclusion

In conclusion, the analytical method is suitable for the quantification of cymoxanil and IN-KQ960 in water matrices at an LOQ of 0.100 ppb

(Cermak J. 2013)

A 2.2.1.11 Description of Methods for the Analysis of Air (KCP 5.2)

No new data.

A 2.2.1.12 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new data.

A 2.2.1.13 Other Studies/ Information

No new data.