REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: AG-E1-500 SC1

Product name: Ethosat 500 SC

Chemical active substances:

Ethofumesate, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Sponsor: ADAMA Agan Ltd.
Applicant: Country organisation / representative of ADAMA, as given in Part A

Submission date: March 2021

MS Finalisation date: January 2022 (initial core Assessment)

June 2022 (final Core Assessment)

Version history

When	What
March 2021	dRR version 1 submitted by applicant.
January 2022	dRR version 1.1 submitted by applicant – validation of analytical method RES-00278 for the determination of ethofumesate and ethofumesate-2-keto residues in lettuce and cereal grain
January 2022	Initial ZRMS assessment. The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are ~~struck through and shaded for transparency~~.
June 2022	Final report (Core Assessment updated following the commenting period). No additional information or assessments after the commenting period.

DATA PROTECTION CLAIM

Under Article 59, Regulation 1107/2009/EC, on behalf of the Sponsor Company 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

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5 Analytical methods

This document summarizes the analytical methods on the plant protection product AG-E1-500 SC1, a suspension concentrate containing 500 g/L ethofumesate for use in sugar beet and fodder beet in Central Zone according to Article 33 of the Regulation 1107/2009.
This application follows the data requirements for the active substance laid down in Regulation (EC) No. 283/2013 for the active substance ethofumesate, and Regulation (EC) No. 284/2013 for the plant protection product AG-E1-500 SC1.

5.1 Conclusion and summary of assessment

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

- None

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.
Noticed data gaps are:

- None

Commodity/crop	Supported/ Not supported
Sugar beet	supported
Fodder beet	supported

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 and possible data gaps for analysis of the active substances in the plant protection product AG-E1-500 SC1 (containing 500 g/L ethofumesate) is provided as follows.

Comments of zRMS:	The analytical method was successfully validated for the determination of ethofumesate in Ethosat 500 SC (AG-E1-500 SC1) formulation according to the requirements laid down by SANCO/3030/99 rev.5.

Reference:	KCP 5.1.1/01, filed under KCP 2.1/01
Report	Determination of Storage Stability and Physical-Chemical Properties of Ethosat 500 SC (AG-E1-500 SC1) Stored at 54°C for 14 Days and at 0°C for 7 Days Tsesin, N. (2020) Report no. 000104496.057FL, 000104496
Guideline(s):	Yes, SANCO/3030/99 rev. 5 (22 March 2019)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The analysis of ethofumesate was done by high performance liquid chromatography (HPLC) coupled with DAD detection using external standard technique.
This analytical method was used for the content determination of ethofumesate during storage stability studies, spontaneity of dispersion and suspensibility testing.

Test material:
Ethofumesate 500 SC (AG-E1-500 SC1), Batch no. F4905, Content: ethofumesate: 517.5 g/L
Matrix Blank, Batch no. BL-F4804

Reference material: Ethofumesate, Batch no. 277-3724, ADAMA Agan Standard Laboratory, Purity: 98.4%.

Sample preparation:

Repeatability: Five weightings, about 100 mg each, of Ethosat 500 SC formulation product (Batch No: F4905) were made into separate 50 ml volumetric flasks. Acetonitrile was added as a solvent and solutions were sonicated for about 5 min. These solutions were filtered prior to measurements through a disposable Nylon filter (0.45 μm) and injected into the HPLC-DAD.

Accuracy:
To three sets of two matrix blank samples containing appropriate amount of material each, Ethofumesate standard was added at maximal, medium and minimal concentration levels in final solutions. A blank, containing about 55 mg matrix blank without spike, was prepared and used to obtain an indication of the contribution of the AI contents in the sample to the overall peak area. Acetonitrile was used as the solvent in samples preparation.

HPLC-DAD Conditions:

HPLC	ThermoFisher Scientific Dionex UltiMate 3000 equipped with an autosampler,column oven and degasser, Diode array detector
Column	Zorbax Eclipse Plus C18, 5µm, 150 x 4.6 mm ID
Column temperature	30°C
Mobile phase	Acetonitrile : 0.1 % v/v phosphoric acid in water, (60:40; v/v)
Injection Volume	5 µL
Flow	1.5 mL/min
Detector	225 nm
Retention time:	Ethofumesate about 3.5 min.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance ethofumesate in plant protection product Ethofumesate 500 SC (AG-E1-500 SC1)

	Ethofumesate (nominal concentration 2 mg/mL)
Author(s), year	Tsesin, N. (2020)
Principle of method	High performance liquid chromatograph (HPLC) with DAD detection
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	Six levels (injected in duplicate) in the range of about 0.4 – 1.4 mg/mL which is about 45% to 155% concentration level) were prepared in acetonitrile. Correlation coefficient: R = 0.9999 Y = 72.5557x+ 0.6379, r^2 = 0.9998
Precision – Repeatability Mean n = 10 samples (%RSD)	RSD = 0.40%
Accuracy n = 2, two samples for each level (% Recovery)	Two preparation spiked at a.i. concentration levels (80%, 100%, 120%)

Mean Recovery (REC), % and RSD %

Level	**120 %**		**100 %**		**80 %**	
	Rec	**RSD**	**Rec**	**RSD**	**Rec**	**RSD**
	99	0.28	101	0.17	101	0.63

Interference/ Specificity	Comparison with matrix blank solution injection Interferences were <3% of the total peak area target analyte
Comment	Acceptable

Conclusion

The analytical method for the active ingredient ethofumesate determination in AG-E1-500 SC1 was fully validated in regard of linearity, precision, accuracy and specificity. Validation acceptance criteria are based on guidance for generation and reporting methods of analysis in support of data requirements of SANCO/3030/99 rev. 5. The method is acceptable.

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

According to Commission Implementing Regulation (EC) 2016/1426 the following impurities are of toxicological concern and must not exceed the following levels in the technical material:

- EMS; ethyl methane sulfonate: maximum content 0.1 mg/kg
- iBMS; iso-butyl methane sulfonate: maximum content 0.1 mg/kg

Therefore, an analytical method for determination of these impurities in the product AG-E1-500 SC1 is provided with this application and summarised in the following. An overview on the acceptable methods and possible data gaps for analysis of relevant impurities in plant protection product is provided as follows:

Comments of zRMS:	The analytical method was successfully validated for the determination of Methanesulfonic Acid Ethyl Ester (EMS) and Methanesulfonic Acid Isobutyl Ester (iBMS) in Ethosat 500 SC formulation according to the requirements laid down by SANCO/3030/99 rev.4.

Reference:	KCP 5.1.1/02
Report	Method Validation for the Determination of Methanesulfonic Acid Ethyl Ester (EMS) and Methanesulfonic Acid Isobutyl Ester (iBMS) in Three Technical Ethofumesate Formulations Bacher R, (2010) PTRL Europe Study/ Report No. P/B 1686 G, 0FC00022182
Guideline(s):	Yes, SANCO/3030/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of this study was to develop and to validate an analytical method for the determination of the impurities methanesulfonic acid ethyl ester (EMS) and methanesulfonic acid isobutyl ester (iBMS) present in Ethosate 500. The target limit of quantification (LOQ) was 0.10 mg/kg per analyte, calculated based on the respective ethofumesate content.

Test material:

Ethosat 500 (FSG 03189 H), Batch No.: F4005, active Ingredient: 476 g/L ethofumesate
Methanesulfonic acid ethyl ester (EMS), Lot No.: 09224PR, Purity: 100 %
Methanesulfonic acid isobutyl ester (iBMS), Lot No.: FC 2205, Purity: 100 %

Sample preparation:

The ethofumesate containing formulation was partitioned with n-hexane. The n-hexane solution was analyzed for the impurity's EMS and iBMS by GC-MS applying external standardization.

GC-MS Conditions:

GC	Thermo TSQ Quantum triple quad GC/MS system, consisting of Trace Ultra gas chromatograph equipped with TriPlus autosampler, split/splitless injector, and TSQ Quantum triple quad mass spectrometer with closed electron impact (EI) ion volume, helium as carrier gas, and XCalibur 2.0 Software.
Autosampler	Basic injection mode with air gap, injection rate 100 µL/sec, Injection volume 3 µL.
Carrier gas	Helium with constant flow at 1.2 mL/min.
Injection technique	Split/splitless injection (sptitless period: 1 min), Injector temperature 220 °C.
GC capillary column	Varian VF-5ms fused silica capillary column (30 m length, 0.32 mm inner diameter, 0.25 um film thickness). Stationary phase: 5 % phenyl 95 % dimethylpolysiloxane.
Oven temperature program	60°C, 2 min hold, ramp with 10°C/min to 130°C, ramp with 50°C/min to 300°C, 4 min hold at 300°C.
Retention time	EMS: approx. 3.4 min (74°C). iBMS: approx. 5.7 min (97°C).
MS detection	Electron impact (EI) ionization and mass spectrometric detection in the selected ion monitoring mode (SIM), emission current: 25 µA. 2.7 min filament/multiplier delay, two acquisition segments. Segment 1 (0-4.5 min): EMS: 79 m/z, 97 m/z and 109 m/z. Segment 2 (4.5-10.9 min): iBMS: 80 m/z, 109 m/z and 111 m/z.
Calibration range	External calibration from e.g. 0.5,0.7 or 1.0 to 50 or 100 ng/mL (> 5 levels, linear regression with 1/x weighting)

GC-MS in the selective ion monitoring (SIM) mode was used for analysis of the impurity's EMS and iBMS. Three fragment ions per analyte were monitored for quantification of the analytes (79 m/z, 97 m/z, 109 m/z for EMS; 80 m/z, 109 m/z, 111 m/z for iBMS). GC-MS, monitoring three fragment ions per analyte, is considered to be highly selective, thus no further confirmation is required.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in Ethosate 500

	Methanesulfonic acid ethyl ester (EMS)	**Methanesulfonic acid isobutyl ester (iBMS)**
Author(s), year	Bacher, R., 2010	
Principle of method	Partitioning with n-hexane and analysis via GC/MS (m/z: 79, 97, 109)	Partitioning with n-hexane and analysis via GC/MS (m/z: 109, 111, 90)
Linearity	Matrix matched standards 1 ng/mL and 100 ng/mL $R \leq 0.99$	Matrix matched standards 1 ng/mL and 100 ng/mL $R \leq 0.99$
Precision – Repeatability Mean n =8 (%RSD)	0.38 % at 47.5 ng/mL Only m/z 109 stated here, worst precision	0.05 % at 47.5 ng/mL Only m/z 111 stated here, worst precision
Accuracy n = 5 (% Recovery)	83 ± 4 % at 0.1 mg/kg 82 ± 9 % at 1.0 mg/kg Only m/z 79 stated here	105 ± 5 % at 0.1 mg/kg 101 ± 6 % at 1.0 mg/kg Only one m/z 109 stated here
Interference/ Specificity	Below 30 % of LOQ	Below 30 % of LOQ
LOQ	0.1 mg/kg	0.1 mg/kg
Comment	Acceptable	Acceptable

Conclusion
The analytical method fulfils the requirements of SANCO/3030/99 rev.4 and is suitable for the determination of methanesulfonic acid ethyl ester (EMS) and methanesulfonic acid isobutyl ester (iBMS) in the plant protection product AG-E1-500 SC1 (Ethosate 500).

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

None of the formulants or their constituents of the plant protection product AG-E1-500 SC1 are considered by the applicant to represent compounds of particular toxicological, ecotoxicological or environmental concern. The submission of analytical methods for such is therefore not considered to be required.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For the active substance Ethofumesate Technical the CIPAC method no. 233/TC/M/- in Handbook J page 44 is available. An ethofumesate suspension concentrate can be analysed using CIPAC method no. 233/SC/M/- and an ethofumesate emulsion concentrate using CIPAC method no. 233/EC/M/- as described in CIPAC Handbook J page 48 - 50.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of ethofumesate for the generation of pre-authorization data is given in the following table. For the detailed evaluation of the submitted new studies it is referred to Appendix 2.

Table 5.2-3: Validated methods for the generation of pre-authorization data for ethofumesate in plant and animal products

Plant residue definition for risk assessment **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)** **Animal residue definition for risk assessment** **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)**				
Matrix type	**Method type and analyte**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Sugar beet tops Sugar beet roots (Residues)	Primary method Method RESID/73/18/1 and RESID/73/18/2 (with some modifications) Ethofumesate NC 8493 NC 9607 NC 8493 NC 20645 (addition of NC 9607)	 0.05 mg/kg 0.02 mg/kg 0.05 mg/kg 0.05 mg/kg 0.05 mg/kg 0.02 mg/kg 0.05 mg/kg 0.10 mg/kg 0.05 mg/kg 0.05 mg/kg	GC-FPD	Whiteoak, R. J.; Crofts, M.; Harris, R. J.; 1973 Report: M-155727-01 and Whiteoak, R. J.; Crofts, M.; Harris, R. J. 1976 Report: M-155728-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-FPD)	
Sugar beet (immature plant) Sugar beet (body) Sugar beet (leaves) (Residues)	Primary method Method RESID/84/42 Ethofumesate NC 8493 (free form) NC 8493 (conjugated form) NC 9607 (free form) NC 20645 (conjugated form)	 No LOQ For all analytes	GC-FPD	Manley, J. D., Snowdon, P.J., 1984 Report M-155729-01-1 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-FPD)	
Peas and sugar beet roots (Residues)	Primary method Method AL 081/96-0 Ethofumesate	 No LOQ	GC-MS	Wrede, A., 2000 Document no: M-199547-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-MS)	
Sugar beets (Residues)	Primary method Method A0019 Ethofumesate, 2-keto ethofumesate	 0.05 mg/kg	GC-MS	Perny, A.,2002 Document No. A0019 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-MS)	

Plant residue definition for risk assessment **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)** **Animal residue definition for risk assessment** **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)**				
Matrix type	**Method type and analyte**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Sugar beet, leaf and body (Residues)	Primary method Method 00955/M002* Ethofumesate, 2-keto ethofumesate	0.01 mg/kg	GC-MS	Konrad S., 2012 Document no: M-438402-01-1 Schulte, G.; 2013; Document no: M-459805-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-MS)	
Sugar beet leaf, sugar beet root (Residues)	Primary method Method 01343 open-ring-2-ketoethofumesate (AE C520645, NC 20645)	0.01 mg/kg	LC-MS/MS	Schulte, G.; 2013 Document no: M-459806-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
Wheat, sunflower seed (Residues)	Primary method Method PR00/001 Ethofumesate, NC 9607 (free form), NC 20645 (free and conjugated form)	0.02 mg/kg	GC-MS	Thom, M.; 2005 Document no: M-351876-01-1 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-MS)	
Lettuce, Cereal grain (barley) (Residues)	Primary method RES-00278 Ethofumesate and ethofumesate-2-keto	0.01 mg/kg	GC-MS	KCP 5.1.2/01 filed under KCP 8/02 (KCA 6.1/03) Watson G., 2021 Report No: RES-00278 Study ID: 000106576 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-MS)	
KCA 4.1.2 /25; [redacted] ;1977; M-155301-01 KCA 4.1.2 /26; Whiteoak, R. J.;1990; Document no: M-155384-01 KCA 4.1.2 /27; [redacted];1975; Document no: M-155288-01 KCA 4.1.2 /28; [redacted] 1999; Document no: M-185949-01				*EU agreed* *First Annex I inclusion, Monograph and base line dossier (D-008920)*
Whole milk (Residues)	Primary method Method XB/01/01 Ethofumesate, NC 9607 NC 20645	 0.01 mg/kg 0.05 mg/kg	GC-FPD	[redacted] 1994; Document no: M237976-01 Cole, M. G.; 2000 Document no: M187353-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (GC-FPD)	

Plant residue definition for risk assessment **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)** **Animal residue definition for risk assessment** **Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate) (EFSA, 2016)**				
Matrix type	**Method type and analyte**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Cattle matrices (Cream, Whey, Milk, Muscle, Fat, Liver, Kidney) (Residues)	Primary method Method AD-001-A10-02 Ethofumesate, NC 9607– measured as NC 20645 – and NC 20645 itself and NC 8493	0.01 mg/kg	LC-MS/MS	[redacted] 2010 Document no: M-388797-01-1 Perez, R.; Schmitt, J. L.; Patel, D., 2014 Document no: M-467206-01 *EU agreed* *RAR Ethofumesate, 2015*
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
Soil, water, sediment,... (Environmental fate)	No additional data.			
	Confirmatory (if required)	--	--	--
Soil, water,... (Efficacy)	No additional data.			
	Confirmatory (if required)	--	--	--
Feed, body fluids (Toxicology)	No additional data.			
	Confirmatory (if required)	--	--	--
Body fluids, air,.... (Exposure)	Primary			
	Confirmatory (if required)			

* Residue analytical method 00955/M002 was developed as a data collection method for the determination of the residues of ethofumesate (parent compound) and the common moiety NC 9607 (which comprises the metabolites NC 9607 and NC 20645 and conjugates of NC 20645) in/on plant matrices.

Table 5.2-4: Validated methods for the generation of pre-authorization data for ethofumesate in soil, water and air

Component of residue definition: **Soil: Ethofumesate and NC 8493** **Ground water, surface water and sediment: Ethofumesate, 2-hydroxy-ethofumesate (NC 8493) and openring-2-keto-ethofumesate (NC 20645)** **Air: Ethofumesate**				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Tap water *(Fish* - Acute toxicity) OECD 203 (Ecotoxicology)	Primary method	Ethosat 500 SC 0.01 mg/L	HPLC-UV	KCP 5.1.2/01 filed under KCP 10.2/01 Scheerbaum D. 2005 Study No: FAG100321 **See Appendix 2**
	Confirmatory	--	Not required, highly specific detection system was used	

Component of residue definition: **Soil: Ethofumesate and NC 8493** **Ground water, surface water and sediment: Ethofumesate, 2-hydroxy-ethofumesate (NC 8493) and openring-2-keto-ethofumesate (NC 20645)** **Air: Ethofumesate**				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
	(if required)		(HPLC-UV)	
ISO Standard water 6341 (*Daphnia magna* – Acute toxicity) OECD 202 (Ecotoxicology)	Primary method	0.7246 mg/L Ethofumesate (correspond to 1.449 mg/L undiluted test solution)	LC-MS/MS	KCP 5.1.2/02 filed under KCP 10.2/02 Renner P. 2020a Study No: 20 48 ADL 0001 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
OECD medium (*Desmodesmus subspicatus* - algal growth inhibition test) OECD 201 (Ecotoxicology)	Primary method	0.959 mg/L Ethofumesate	LC-MS/MS	KCP 5.1.2/~~02~~ 03 filed under KCP 10.2/03 Renner P. 2020b Study No: 20 48 AAL 0001 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
Smart and Barko medium / sediment (*Myriophyllum spicatum L.)* OECD 239 (Ecotoxicology)	Primary method	2.379 µg/L Ethofumesate	LC-MS/MS	KCP 5.1.2/04 filed under KCP 10.2/04 Renner P. 2020c Study No: 20 48 AMS 0001 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
50% aqueous sucrose solution, dose verification - Chronic Honey Bee study (*Apis mellifera L.*) OECD 245 (Ecotoxicology)	Primary method	374 mg test item/kg (171 mg ethofumesate/kg)	QuEChERS method LC-MS/MS	KCP 5.1.2/05 filed under KCP 10.3.1.2/01 Ansaloni T. 2020a Study No: S19-20080 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
Larval diet C, dose verification – Honey Bee (*Apis mellifera L.*) OECD 239 (Ecotoxicology)	Primary method	53.4 mg test item/kg (24.4 mg ethofumesate/kg)	QuEChERS method LC-MS/MS	KCP 5.1.2/06 filed under KCP 10.3.1.3/01 Ansaloni T. 2020b Study No: S19-20081 **See Appendix 2**
	Confirmatory (if required)	--	Not required, highly specific detection system was used (LC-MS/MS)	
Seedling Emergence and Seedling Growth – aqueous solution (Ecotoxicology)	Primary method	144 µg ai/mL	LC-UV	KCP 5.1.2/07 filed under KCP 10.6.2/01 Duffner A. 2020a Study No: S19-22437 **See Appendix 2**
	Confirmatory	--	Not required, specific detection system was used (LC-UV)	

Component of residue definition: **Soil: Ethofumesate and NC 8493** **Ground water, surface water and sediment: Ethofumesate, 2-hydroxy-ethofumesate (NC 8493) and openring-2-keto-ethofumesate (NC 20645)** **Air: Ethofumesate**				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
	(if required)			
Vegetative Vigour – aqueous solution (Ecotoxicology)	Primary method	144 µg ai/mL	LC-UV	KCP 5.1.2/08 filed under KCP 10.6.2/02 Duffner A. 2020b Study No: S19-22438 **See Appendix 2**
	Confirmatory (if required)	--	Not required, specific detection system was used (LC-UV)	
Water, buffer solutions,... (Properties)	No additional data.			
	Confirmatory (if required)	--	--	--

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Please refer to the analytical methods for the determination of the active substances in the plant protection product as provided in chapter 5.2.1.

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

For this application, it is referred to the following EU concluded residue definitions:

5.3.2.1 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 5.3-1: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate)	0.03 mg/kg	Commission Regulation (EU) 2017/1016 of 14 June 2017
Plant, high acid content			
Plant, high protein/high starch content (dry commodities)			
Plant, high oil content			
Plant, difficult matrices (tea, coffee beans, hops, spices)		0.1 mg/kg	
Muscle	Ethofumesate (Sum of ethofumesate, 2-keto-ethofumesate (NC 9607), open-ring-2-keto-ethofumesate (NC 20645) and its conjugate, expressed as ethofumesate)	0.03 mg/kg	
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	Ethofumesate	0.05 mg/kg	Common limit acc to SANCO/825/00 rev. 8.1
Drinking water (Human toxicology)	Ethofumesate	0.1 µg/L	General limit for drinking water
Surface water (Ecotoxicology)	Ethofumesate	Chronic (flow-through), FFLC Growth, NOEC 0.156 mg a.s./L (mm)	EFSA Journal 2016;14(1):4374
Air	Ethofumesate	0.5 µg/m3	AOEL of 2.5 mg/kg body weigh per day (dog 90 days)
Tissue (meat or liver)	Ethofumesate	0.01 mg/kg	EFSA Journal 2016;14(1):4374 Not classified as T / T+
Body fluids			

zRMS comments:
Proposed uses for Ethosat 500 SC: sugar beet, fodder beet.
Residues definition:
- for plants

According to the EFSA Journal 2016;14(1):4374 and Regulation 2017/1016: Ethofumesate, ethofumesate-lactone

(NC 9607), ethofumesate-carboxylic acid (NC 20645) and its conjugate (their sum expressed as ethofumesate)

- for foodstuff of animal origin

According to the EFSA Journal 2016;14(1):4374: Ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) (their sum expressed as ethofumesate)
According to the Regulation 2017/1016: Ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) and its conjugate (their sum expressed as ethofumesate)

The value of MRLs (Regulation 2017/1016):
- 0.2 mg/kg (sugar beet roots, beetroots),
- 0.03 mg/kg* (foodstuff of animal origin).

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 for analysis of ethofumesate in plant matrices is given in the following tables. No new or additional studies were submitted.

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

Component of residue definition: Sum of ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) and its conjugate, expressed as ethofumesate				
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 High acid content High oil content High protein/high starch content (dry)	Primary	0.01 mg/kg	LC-MS/MS	Schulte, G., Diehl, P.; 2014 MR-13/101 *EU agreed (EFSA Journal 2016;14(1):4374)*
	ILV	0.01 mg/kg	LC-MS/MS	Ingham, R.; 2014, Report No. M-475932-01-1 *EU agreed (EFSA Journal 2016;14(1):4374*)
	Confirmatory (if required)	-	Not required, highly specific detection system was used (LC-MS/MS).	

For any special comments or remarkable points concerning the analytical methods for the determination of residues in plant matrices, please refer to Appendix 2.

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	No residues (according to the residue definition; refer to chapter B.7) above the LOQ are present in food commodities (representative use: sugar beets). Therefore, there is no need to address extraction efficiency. Reference to guidance document SANCO/825/00 rev.8.1. (see dRAR of Ethofumesate Volume 3 – B.5 (AS)) Common moiety method was used by Schulte, G., Diehl, P.; 2014, MR-13/101 Expected Residues: > LOQ Sugar beet tops < LOQ for Sugar beet roots

zRMS comments:
Sufficient analytical method for the determination of ethofumesate (according to the residue definition) residues in crops (Schulte, G.; Diehl, P.; 2014; Method 01392) and its ILV (Betson, S.; 2014) is available (RAR, 2015). The method has been validated with LOQ=0.01mg/kg by LC-MS/MS for ethofumesate, NC 9607 as NC 20645 and NC 20645 separately in high water content, dry, fatty, acidic and no group (hop) commodities. As the method is highly specific (two mass transitions), confirmatory method is not required.

Extraction efficiency of the method and efficiency of the acidic hydrolysis have been demonstrated for high water content commodities in the RAR of ethofumesate (2015).
No additional data are required.

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

An overview on the acceptable methods and possible data gaps for analysis of ethofumesate in animal matrices is given in the following tables. No new or additional studies were submitted.

Table 5.3-4: Validated methods for food and feed of animal origin

Component of residue definition: Ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) (their sum expressed as ethofumesate)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk, Eggs, Muscle, Fat, Kidney, Liver	Primary	0.01 mg/kg	LC-MS/MS	Jooß S., 2012, P 2371 G *EU agreed (EFSA Journal 2016;14(1):4374)*
	ILV	0.01 mg/kg	LC-MS/MS	Schlewitz, P. 2013b, R B1218 *EU agreed (EFSA Journal 2016;14(1):4374)*
	Confirmatory (if required)	-	Not required, highly specific detection system was used (LC-MS/MS).	

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	No residues (according to the residue definition; refer to chapter B.7) above the LOQ are expected in food of animal commodities regarding the representative use: sugar beets. Therefore, there is no need to address extraction efficiency. Reference to guidance document SANCO/825/00 rev.8.1. (see dRAR of Ethofumesate Volume 3 – B.5 (AS))

zRMS comments:
Sufficient analytical method for the determination of ethofumesate and its two metabolites NC 9607 (2-ketoethofumesate) and NC 20645 (2-methylpropionic acid ethofumesate) in various animal matrices (Jooß, S. (2012), P 2371 G) and its ILV (Schlewitz, P. (2013b), R B1218) is available (RAR, 2015). The method has been validated with a limit of quantitation (LOQ) of 0.01 mg/kg per analyte, always expressed as Ethofumesate by LC-MS/MS. As the method is highly specific (two mass transitions), confirmatory method is not required.
No residues (according to the residue definition; refer to chapter B.7) above the LOQ are expected in food of animal commodities regarding the representative use: sugar beets. Therefore there is no need to address extraction efficiency.
According to the Regulation 2017/1016 the residue definition for monitoring purposes for animal matrices: Ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) and its conjugate (their sum expressed as ethofumesate).
This method (Jooß, S. (2012), P 2371 G) does not include a hydrolysis step, so conjugates are not quantified in this method. However, as it is stated in RAR (2015), *"no residues (according to the residue definition) above the LOQ are expected in food of animal commodities regarding the representative use: sugar beets"*. Therefore, no further data is required for the registration of Ethosat 500 SC.

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

An overview on the acceptable methods for analysis of ethofumesate in soil is given in the following tables. No new or additional studies were submitted.

Table 5.3-6: Validated methods for soil

Component of residue definition: Ethofumesate			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.05 mg/kg	LC-MS/MS one transitions	Brumhard, B; 2003, 00806 *EU agreed (EFSA Journal 2016;14(1):4374)*
Confirmatory	0.05 mg/kg	GC-MS	Schneider, E.; 2000, OFC00004917 *EU agreed (EFSA Journal 2016;14(1):4374)*

zRMS comments:
Sufficient analytical method is available for the determination of ethofumesate in soil (RAR, 2015). The LC-MS/MS method 00806 has been sufficiently validated in soil (LOQ = 50 µg/kg or 0.05 mg/kg). Since only one transition has been reported for method 00806, an additional GC-MS method (PR00/003) has been provided for first Annex I inclusion and submitted for confirmation.
No additional data are required.

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

An overview on the acceptable methods for analysis of ethofumesate in surface and drinking water is given in the following tables. No new or additional studies were submitted.

Table 5.3-7: Validated methods for water

Component of residue definition: Ethofumesate				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	-	-	Not required, covered with surface water validation data
	ILV	-	-	
	Confirmatory	-	-	
Surface water	Primary	0.05 µg/L	HPLC-MS/MS	Krebber, R.; Braune, M., 2013, MR-13/085 *EU agreed (EFSA Journal 2016;14(1):4374)*
	ILV	0.05 µg/L	HPLC-MS/MS	Class, T., Stanislowski T., 2013, P3117 G *EU agreed (EFSA Journal 2016;14(1):4374)*
	Confirmatory (if required)	-	Not required, highly specific detection system was used (HPLC-MS/MS).	

zRMS comments:
Sufficient analytical method (Krebber, R.; Braune, M., 2013, MR-13/085) and its ILV (Class, T., Stanislowski T., 2013, P3117 G) is available for the determination of ethofumesate in surface water (RAR, 2015). The LC-MS/MS method 01387 has been sufficiently validated in surface water with a limit of quantitation (LOQ) of 0.05 µg/L. As the method is highly specific (two mass transitions), confirmatory method is not required.

No additional data are required.

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

An overview on the acceptable methods and possible data gaps for analysis of ethofumesate in air is given in the following tables. No new or additional studies were submitted.

Table 5.3-8: Validated methods for air

Component of residue definition: Ethofumesate			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.5 µg/m3	GC-MS	Schneider, E.; 2000; OFC00004919 EU agreed (EFSA Journal 2016;14(1):4374)
Confirmatory (if required)	-	Not required, highly specific detection system was used (GC-MS)	

zRMS comments:
Sufficient analytical method (Schneider, E.; 2000; OFC00004919) is available for the determination of ethofumesate in air (RAR, 2015).
The GC-MS method has been sufficiently validated in air with a limit of quantitation (LOQ) of 0.5 µg/m^3. As the method is highly specific (two mass transitions), confirmatory method is not required.
No additional data are required.

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

An overview on the acceptable methods and possible data gaps for analysis of ethofumesate in body fluids and tissues is given in the following table. No new or additional studies were submitted.

Table 5.3-9: Methods for body fluids and tissues

Component of residue definition: Ethofumesate			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.1 mg/L	HPLC-UV	McKenzie 1998, A87557 *EU agreed (EFSA Journal 2016;14(1):4374)*
Confirmatory (if required)	-	Not required, specific detection system was used (HPLC-UV).	

zRMS comments:
Accoridng to the RAR (2015) *analytical methods are available for animal matrices including tissues (meat) and fluids (milk) in this DRAR (Jooß S., 2012) and is as well addressed for dog plasma (McKenzie 1994) in the original DAR (1998).*
Analytical method (McKenzie, 1994) for the determination of ethofumesate residues in body fluids is available and has been validated by HPLC-UV with LOQ=0.1mg/L for ethofumesate in dog plasma (RAR, 2015).
Analytical method Jooß, S. (2012) is available and has been validated by LC-MS/MS with LOQ=0.01mg/kg for ethofumesate, NC 9607 and NC20645 (free) separately in meat, egg, fat, milk, liver, kidney. As the method is highly specific confirmatory method is not required.
No additional data are required.

5.3.2.8 Other studies/ information

No new or additional studies were submitted.

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 5.1.1/01	Tsesin, N.	2020	Determination of Storage Stability and Physical-Chemical Properties of Ethosat 500 SC (AG-E1-500 SC1) Stored at 54°C for 14 Days and at 0°C for 7 Days ADAMA Agan Ltd. Israel, Report No. 000104496.057FL, Sponsor reference no. 000104496 GLP Unpublished Also filed under KCP 2.1/01	N	ADM
KCP 5.1.1/02	Bacher R.	2010	Method Validation for the Determination of Methanesulfonic Acid Ethyl Ester (EMS) and Methanesulfonic Acid Isobutyl Ester (iBMS) in Three Technical Ethofumesate Formulations Report No. P/B 1686 G, Sponsor reference no. 0FC00022182 PTRL Europe, Ulm, Germany GLP Unpublished	N	ADM
KCP 5.1.2/01	Watson, G.	2021	Ethofumesate: Storage Stability of Residues of Ethofumesate and its Metabolite in Lettuce and Cereal Grain Stored Frozen for up to Two Years; Interim Report 1. Report No. RES-00278 Study ID 000106576 GLP Unpublished Also filed under KCP 8/02 (KCA 6.1/03)	N	ADAMA
KCP 5.1.2/02	Scheerbaum D.	2005	Ethosat 500 - Fish (Golden Orfe), Acute Toxicity Test, Semi-Static, 96 h Report No. FAG100321 Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished Also filed under KCP 10.2/01	Y	ADM
KCP 5.1.2/03	Renner P.	2020a	Acute toxicity of AG-E1-500 SC1 to *Daphnia magna* in a 48-hour static test Report No. 20 48 ADL 0001, ADAMA reference no. 000103254 BioChem agrar, Machern OT Gerichshain, Germany GLP Unpublished	N	ADM

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**
			Also filed under KCP 10.2/02		
KCP 5.1.2/04	Renner P.	2020b	Effects of AG-E1-500 SC1 on *Desmodesmus subspicatus* in an algal growth inhibition test Report No. 20 48 AAL 0001, ADAMA reference no. 000103255 BioChem agrar, Machern OT Gerichshain, Germany GLP Unpublished Also filed under KCP 10.2/03	N	ADM
KCP 5.1.2/05	Renner P.	2020c	Effects of AG-E1-500 SC1 on *Myriophyllum spicatum* in a static water-sediment system Report No. 20 48, ADAMA reference no. AMS 0001 000103256 BioChem agrar, Machern OT Gerichshain, Germany GLP Unpublished Also filed under KCP 10.2/04	N	ADM
KCP 5.1.2/06	Ansaloni T.	2020a	AG-E1-500 SC 1: Chronic Oral Toxicity Test (10-Day Feeding) to the Honey Bee, *Apis mellifera* L. under Laboratory Conditions Report No. S19-20080, Sponsor reference no. 000103264 Trialcamp S.L.U., Alcàsser, Spain GLP Unpublished Also filed under KCP 10.3.1.2/01	N	ADM
KCP 5.1.2/07	Ansaloni T.	2020b	AG-E1-500 SC 1: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions Report No. S19-20081, Sponsor reference no. 000103265 Trialcamp S.L.U., Alcàsser, Spain GLP Unpublished Also filed under KCP 10.3.1.3/01	N	ADM
KCP 5.1.2/08	Duffner A.	2020a	AG-E1-500 SC1: Effects on the Seedling Emergence and Seedling Growth of Non-Target Terrestrial Plant Speciedunder Greenhouse Conditions Report No. S19-22437, Sponsor reference no. 000104143 Eurofins Agroscience Services Ecotox GmbH, Germany GLP Unpublished Also filed under KCP 10.6.2/01	N	ADM

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	Vertebrate study Y/N	Owner
KCP 5.1.2/09	Duffner A.	2020b	AG-E1-500 SC1: Effects on the Vegetative Vigour of Non-Target Terrestrial Plant Specied under Greenhouse Conditions Report No. S19-22438, Sponsor reference no. 000104144 Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany GLP Unpublished Also filed under KCP 10.6.2/02	N	ADM

* The sponsor company ADAMA Agan Ltd. is a member of ADAMA Agricultural Solutions.

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

The list below was not validated by the zRMS since majority of the active substance data have been taken from the EU review of Ethofumesate and the complete list of studies evaluated at the EU level is provided in Vol. 2 of the RAR (2015).

Ethofumesate: For data protected studies owned by Bayer the applicant has a Letter of Co-ownership. For studies owned by the TFE (TaskForce Ethofumesate), ADAMA Agricultural Solutions and all its affiliates has also access as ADAMA Deutschland GmbH is member of the TFE. For studies owned by UPL the TFE has either equivalent studies or a Letter of Access.

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	Previous evaluation (DAR) or Renewal
CP 5.1.2	Whiteoak R. J., Crofts M., Harris, R.J.	1973	ANALYTICAL METHOD FOR RESIDUE IN SUGAR BEET TREATED WITH NORTRON Fisons plc, United Kingdom Bayer CropScience, Report No.: A83491/ M-155727-01, Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/28
CP 5.1.2	Whiteoak, R. J.; Crofts, M.; Harris, R. J.	1976	ANALYTICAL METHOD FOR RESIDUES IN SUGAR BEET TREATED WITH NORTRON Fisons plc, United Kingdom Bayer CropScience, EPA MRID No.: 00084997 Report number: A83492/ M-155728-01 Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/29
CP 5.1.2	Manley, J. D., Snowdon, P.J.,	1984	ANALYTICAL METHOD FOR RESIDUES OF ETHOFUMESATE AND MAJOR METABOLITES IN SUGAR BEET (IMPROVED METHOD) FBC Limited, Chesterford Park, United Kingdom Bayer CropScience, Report number: A83493/M-155729-01-1 Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/30
CP 5.1.2	Wrede, A.;	2000	Validation of the method AL 081/96-0 in peas and sugar beet roots by GC-MSD - ethofumesate - Code: AE B049913 Report No: C009934, Document no: M-199547-01-1 Aventis CropScience GmbH, Germany GLP, unpublished	N	Bayer Crop Science	KCA 4.1.2/18, KCA 4.2/04

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	Previous evaluation (DAR) or Renewal
CP 5.1.2	Perny A.	2002	Validation of the Method of Analysis of the residues of Ethofumesate and its metabolite 2-keto ethofumesate (free and conjugated form) in Sugar Beet Report number: A0019 GLP	N	UPL	RAR 2015, Volume 3, B5
CP 5.1.2	Konrad S.	2012	Analytical method 00955/M002 for the determination of ethofumesate and its metabolite AE C509607 in three different plant groups (sugar beet, leaf and body and orange) Currenta GmbH & Co. OHG, Leverkusen, Germany Bayer CropScience, Report No.: 00955/M002 / M-438402-01-1 GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, B5
CP 5.1.2	Schulte G.	2013	Formation of 2-keto-ethofumesate (AE C509607) by acidic extraction of plant matrices containing open-ring-2-keto-ethofumesate (AE C520645) - (sugar beet (leaf), sugar beet (body), orange (fruit), wheat (grain)) Bayer CropScience, Report No.: MR-13/061/ M-459805-01 GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, B5
CP 5.1.2	Schulte G.	2013	STORAGE STABILITY OF OPEN-RING-2-KETO ETHOFUMESATE (AE C520645) IN PLANT MATRICES FOR 24 MONTHS - PHASE REPORT AFTER 6 MONTHS Bayer CropScience Report No: MR-13/086, M-459806-01 GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 6.1
CP 5.1.2	Thom M.	2005	Validation of an analytical method for the determination of residues of ethofumesate and ethofumesate-2-keto in various plant commodities Report No.: OFC00004832, M-351876-01-1 GAB Analytik GmbH, Germany GLP, unpublished	N	ADAMA	RAR 2015, Volume 3, KCA 4.2/22
CP 5.1.2	[redacted]	1977	RESIDUES IN MILK AND TISSUES FOLLOWING A 28-DAY FEEDING STUDY WITH ETHOFUMESATE IN DAIRY COWS - PART 1 Huntingdon Research Centre Ltd., Huntingdon, United Kingdom Bayer CropScience, Report number: A83024, M-155301-01-1	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/25

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**	**Previous evaluation (DAR) or Renewal**
			Non GLP, unpublished			
CP 5.1.2	Whiteoak, R. J	1990	GAS LIQUID CHROMATOGRAPHIC DETERMINATION OF RESIDUES OF ETHOFUMESATE AND ITS METABOLITES IN MILK AND CATTLE TISSUES Schering AG, Berlin, Germany Bayer CropScience Report no: A83109, M-155384-01-1 GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/26
CP 5.1.2	[redacted]	1975	INVESTIGATION OF TISSUE AND EGG RESIDUES FROM HENS FOLLOWING DIETARY INTAKE OF NC 8438 FOR 21 DAYS Fisons plc, United Kingdom Bayer CropScience, Report no: A83011, M-155288-01-1 Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/27
CP 5.1.2	[redacted]	1999	Review of analytical methodology for residues in edible animal products (dairy, tissues, fat and offal) Ethofumesate AE B049913 AgrEvo UK Crop Protection Ltd., Chesterford Park, United Kingdom Bayer CropScience, Report no: C003328, M-185949-01-1 Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998, KCA 4.1.2/28
CP 5.1.2	[redacted]	1994	Ethofumesate-derived residues in the meat and milk of dairy cows: resulting from oral ingestion of ethofumesate AgrEvo USA Company, Residue Chemistry, Pikeville, NC, USA Bayer CropScience, Report No: B002201/M-237976-01 Non GLP, unpublished	N	Bayer CropScience	Original DAR 1998
CP 5.1.2	Cole M.G.	2000	Validation of an analytical method for the residues of NC 20645 in sugar beet roots and whole milk, USA, 1998 Code: AE C639175 00 1B97 0001 Aventis CropScience USA LP, Residue Chemistry, Pikeville, NC, USA Bayer CropScience Report No: C004116/M-187353-01 Non GLP	N	Bayer CropScience	Original DAR 1998

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	Previous evaluation (DAR) or Renewal
CP 5.1.2	[redacted]	2010	Ethofumesate - Magnitude of the residue in dairy cow Bayer CropScience LP, Stilwell, KS, USA Bayer CropScience Report No.: RAADP014/M-388797-01-1 GLP, unpublished	N	Bayer CropScience	Original DAR 1998
CP 5.1.2	Perez R., Schmitt J.L., Patel D.	2014	FREEZER STORAGE STABILITY OF ETHOFUMESATE IN ANIMAL MATRIX SAMPLES - INTERIM REPORT Bayer Crop Science Report No.: M-467206-01, RAADP031 GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 6.1
CP 5.2	Schulte G., Diehl P.	2014	Validation of the analytical method 01392 for the determination of the relevant ethofumesate metabolites in plant matrices by HPLC-MS/MS Bayer CropScience, Report. number: MR-13/101, M-479926-01 GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3
CP 5.2	Ingham R.	2014	Letter of access - Regulation (EC) No. 1107/2009 - Active substance - Ethofumesate post Annex I inclusion - Letter of access from UPL to protected data United Phosphorus Limited, Cheshire, United Kingdom TF- Ethofumesate, Report No.: M-475932-01-1, GLP: n.a., unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3
CP 5.2	Spiegel K.	2014	Ethofumesate - Discussion on the usability of plant enforcement method 01392 for metabolite AE C520645 in matrices with high oil content Report No.: M-497717-01 Non GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3
CP 5.2	Jooß S.	2012	Ethofumesate - Validation of an Analytical Method for the Determination of the Ethofumesate and its two Metabolites NC 9607 and NC 20645 in Foodstuffs of Animal Origin Report No.: P 2371 G PTRL Europe, Ulm, Germany GPL, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 4.2/03

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	Previous evaluation (DAR) or Renewal
CP 5.2	Schlewitz P.	2013b	Independent laboratory validation of an analytical method for the analysis of Ethofumesate and its two metabolites NC 9607 and NC 20645 in foodstuffs of animal origin Report No.: R B1218 Anadiag S.A. Haguenau, France GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 4.2/04
CP 5.2	Brumhard B.	2003	Method 00806 for the determination of residues of Ethofumesate in soil by HPLC-MS/MS Report No.: 00806, M-122176-01-1 Bayer CropScience GLP, unpublished	N	Bayer CropScience	RAR 2015, Volume 3, KCA 4.2/26
CP 5.2	Schneider E.	2000	PR00/003 - Confirmation method for the determination of residues of ethofumesate in soil Report No. OFC00004917, M-351953-01-1 Dr Krebs Analytik, Koeln, Germany GLP, unpublished	N	ADAMA	RAR 2015, Volume 3, KCA 4.2/15
CP 5.2	Krebber R., Braune M.	2013	Analytical method 01387 for the determination of various pesticides in drinking and surface water by HPLC-MS/MS Report No.: MR-13/085, M-466732-01-1 Bayer CropScience GLP, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 4.2/27
CP 5.2	Class T. Stanislowski T.	2013	Independent laboratory validation of BCS analytical methods 01333 and 01387 for determination of various pesticides in surface water by Di-HPLC-MS/MS Report No.: P3117 G PTRL Europe, Ulm, Germany GPL, unpublished	N	Task Force Ethofumesate	RAR 2015, Volume 3, KCA 4.2/28
CP 5.2	Schneider E.	2000	PR00/02 – Validation of an analytical method for the determination of residues of ethofumesate in air – Monitoring method Report No.: OFC00004919 UCL GmbH, Koeln, Germany GLP, unpublished	N	ADAMA	RAR 2015, Volume 3, KCA 4.2/16
CP 5.2	[redacted]	1994	Ethofumesate: Oral (Capsule/Gavage) maximum tolerated dose (MTD) and 28 day repeat dose rangefinding study in dog	Y	Task Force Ethofumesate	Original DAR 1998, KCA

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**	**Previous evaluation (DAR) or Renewal**
			[redacted] Report No.: A87557 Bayer CropScience GLP, unpublished			5.3.1

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 the active substance ethofumesate

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

New studies have been submitted where necessary.
All method validation for pre-registration methods are filed under this data point.

Statement on the analytical method for the determination of ethofumesate and its metabolites in residue studies:

1. Definition of residues in plants for monitoring and risk assessment

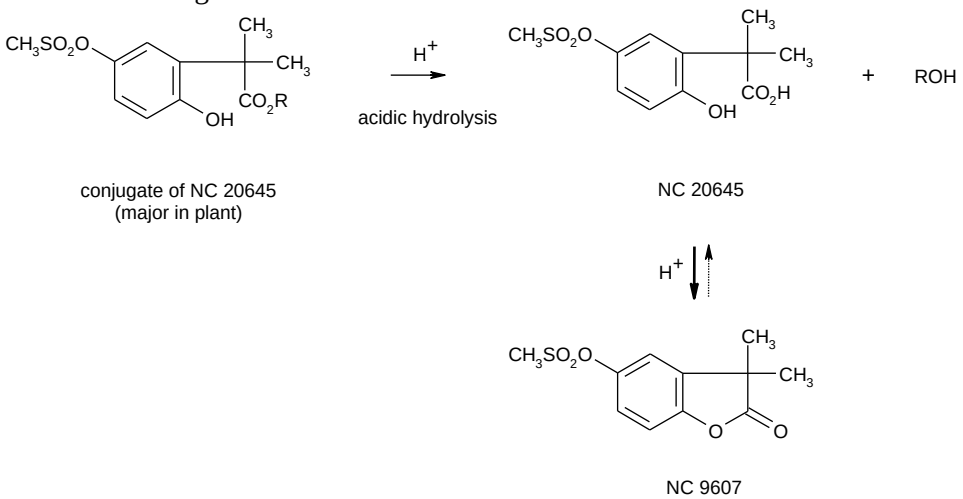
The residue definition for ethofumesate in plant commodities for monitoring and risk assessment has been proposed in the EFSA conclusion published 19 January 2016 (doi:10.2903/j.efsa.2016.4374) as following: Sum of ethofumesate, ethofumesate-lactone (NC 9607), ethofumesate-carboxylic acid (NC 20645) and its conjugate, expressed as ethofumesate.
(or: Sum of ethofumesate, 2-keto–ethofumesate, open-ring-2-keto-ethofumesate and its conjugate, expressed as ethofumesate - as expressed in SANCO/11739/2013 and Commission Regulation (EU) ~~2016/1016~~ 2017/1016).

2. Suitability of Analytical Methods for Analysis of Ethofumesate and its Metabolites according to the proposed residue definition

With reference to the RAR, point B.7.3.1, Tandy, R. (2012b; KCA 6.3.1/15) and of Weir, A. (2014; KCA 6.3.1/16), it was concluded that residue trials can be used to support the residue definition if they were done using acidic conditions and GC/MS analysis.

The rationale behind this conclusion is as follows:
Under acidic conditions conjugated NC20645 (= conjugated open-ring-2-keto, ethofumesate-carboxylic acid) is transformed into free NC20645 (= open ring 2-keto-ethofumesate). This open-ring 2-keto-ethofumesae however always is in chemical equilibrium with its tautomer, which is the closed form, the 2-keto-Ethofumensate (which chemically is a lactone). Under acidic conditions the open-ring 2-keto-ethofumesate is completely and immediately transformed into the closed form, the 2-keto-ethofumesate, which then is the analysed form.

See the following scheme:



NC 9607 is formed nearly quantitatively by acidic hydrolysis of the major plant metabolite, the conjugate of NC 20645. So by using these specific method conditions, the open ring 2-keto-ethofumesate and its conjugate are analysed intrinsically by analysing the 2-keto-ethofumesate.

The analytical method used in the following residue studies are performed under acid conditions. Therefore, the residues of ethofumesate and ethofumesate-2-keto (NC 9607) as determined in the below cited residue studies also cover the residues of the open-ring-2-keto-ethofumesate and its conjugate.
The residue studies and the corresponding methods are in line with the proposed residue definition.

For further information about the method, please refer to the RAR, point B.5, studies Schulte, G.; Diehl, P. (2014), Betson, S. (2014) and Spiegel, K. (2014).

Residue analytical methods

Comments of zRMS:	Analytical method RES-00278 has been successfully validated for the determination of residues of ethofumesate and ethofumesate-2-keto residues in lettuce and cereal grain at a LOQ of 0.01 mg/kg. Acceptable mean recoveries in the range 70 – 110% and a relative standard deviation (RSD) of less than 20% were found for ethofumesate and ethofumesate-2-keto at each fortification level and for both primary and confirmatory transitions in for each matrix type. No residues of ethofumesate or ethofumesate-2-keto greater than 30% of the LOQ were detected in any of the control samples. This method meets the requirements of SANCO/3029/99 rev. 4. The study is acceptable.

Reference:	KCP 5.1.2/01 (filed under KCP 8/02 (KCA 6.1/02)
Report	Watson G., 2021: Ethofumesate: Storage Stability of Residues of Ethofumesate and its Metabolite in Lettuce and Cereal Grain Stored Frozen for up to Two Years, Report No: RES-00278, Study ID: 000106576
Guideline(s):	SANCO/3029/99 rev. 4 and SANTE/2020/12830, Rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The analytical method RES-00278 for the determination of ethofumesate and ethofumesate-2-keto residues in lettuce and cereal grain was validated. The method involves extraction by reflux using ethyl acetate / n-hexane (1/1, v/v). Extracts are concentrated and the retained matrix is then refluxed under acidic conditions before filtration and concentration. Extracts from both reflux steps are combined and concentrated to dryness. Extracts are re-dissolved in n-hexane and passed through a silica gel and C18 cartridge before reducing to dryness and re-dissolving in ethyl acetate. Quantification of ethofumesate and ethofumesate-2-keto is achieved by GC-MS monitoring of three fragment ions for each analyte. The limit of quantification (LOQ) of the method was 0.01 mg/kg.

Test substance:

Test substance	Ethofumesate reference material
Batch No.	226119
Purity	98.6%
CAS No.	26225-79-6
Reference substance:	Ethofumesate-2-keto, reference material
Batch No.	1095584
Purity	98.6%
CAS No.	26244-33-7

Control lettuce was purchased from a local supermarket and was homgenised using a Robot Coupe R10 cutter mixer to produce a composite sample for method validation.
Control cereal grain (barley grain) was purchased from an online supplier and was homogenised using a Fritsch Pulverisette 19 cutting mill to produce a composite sample for method validation.

Homogenised sub-samples of each test commodity (10 g) were fortified with standard solutions of ethofumesate and ethofumesate-2-keto in ethyl-acetate. Five samples of each matrix were fortified at the limit of quantification (LOQ; 0.01 mg/kg) and five at a higher level (10x LOQ; 0.1 mg/kg). Matrices used were lettuce and cereal grain. The fortified samples were analysed alongside untreated control samples and reagent blank.

Analytical method:

Method type	GC-MS
Equipment	Agilent Technologies 6890N Gas Chromatograph coupled to an Agilent 5973 inert Mass Spectrometer with an Agilent 7683 autosampler
Column	30 m x 0.25 mm i.d, HP-5 (5% Phenyl Methyl Siloxane), 0.25µm df
Injection Mode	Pulsed Splitless (30 psi/1 min)
Carrier Gas	Helium, 1.0 ml/min constant flow
Injection Volume	2.0 microlitres
Temperatures	Injector Temperature 270 ºC Transfer Line Temperature 300 ºC Source temperature 230 ºC Quad temperature 150 ºC
Expected retention times (approx.)	Ethofumesate 12.2 mins, ethofumesate-2-keto 11.5 mins
Oven temperature program	Rate (°C/min(/ Temperature (°C) / Time (min) - / 100 / 1.0 10 / 260 / 1.0 30 / 300 / 5.0
Mass Spectrometric Parameters for Ethofumesate and Ethofumesate-2-keto	
Ionization mode	Electron impact (EI)
Dwell	15 msec
Ethofumesate	Primary ion 286 m/z 1 Confirmatory ion 1 207 m/z 1 Confirmatory ion 2 161 m/z
Ethofumesate-2-keto	Primary ion 256 m/z Confirmatory ion 1 177 m/z Confirmatory ion 2 121 m/z

Results and discussions

Recoveries of ethofumesate and ethofumesate-2-keto obtained from lettuce and cereal grain at each fortification level using method RES-00278 are presented in the tables below. Other validation parameters of the method are presented in the following table.

Table A 1: Recovery results from method validation of ethofumesate and ethofumesate-2-keto using the analytical method RES-00278 in lettuce and cereal grain

Matrix	Analyte	Fortification level (mg/kg)	Individual recoveries (%)	Range of recoveries (%) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Reagent blank		NOP**	-	-	-	-	-
Control		NOP**	-	-	-	-	-
Control		NOP**	-	-	-	-	-
Lettuce head	Ethofumesate	Primary ion 286 m/z					
		0.01*	119, 102, 96, 101, 95	96 – 119 (n = 5)	103	9.6	-

Matrix	Analyte	Fortification level (mg/kg)	Individual recoveries (%)	Range of recoveries (%) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
		0.1	92, 92, 91, 91, 92	91 –92 (n = 5)	92	0.7	-
		Overall		91 - 119 (n = 10)	97	9.0	-
		Confirmatory ion 207 m/z					
		0.01*	111, 87, 85, 85, 92	85 - 111 (n = 5)	92	12.1	-
		0.1	102, 101, 99, 99, 101	99 - 102 (n = 5)	100	1.2	-
		Overall		85 - 111 (n = 10)	96	9.0	-
		Confirmatory ion 161 m/z					
		0.01*	120, 101, 100, 101, 96	96 - 120 (n = 5)	104	9.0	-
		0.1	89, 90, 88, 87, 89	97 - 90 (n = 5)	89	1.2	-
		Overall		96 - 120 (n = 10)	96	10.5	-
Cereal grain	Ethofumesate	Primary ion 286 m/z-					
		0.01*	98, 97, 95, 100, 100	95 - 100 (n = 5)	98	2.1	-
		0.1	92, 94, 92, 90, 90	90 - 94 (n = 5)	91	2.0	-
		Overall		90 - 100 (n = 10)	95	4.2	-
		Confirmatory ion 207 m/z					
		0.01*	98, 94, 94, 94, 91	91 - 98 (n = 5)	90.0	3.5	-
		0.1	89, 91, 89, 87, 87	87 - 91 (n = 5)	93.1	7.9	-
		Overall		(n = 10)	91.5	6.1	-
		Confirmatory ion 161 m/z					
		0.01*	96, 93, 90, 90, 89	89 - 96 (n = 5)	92	3.2	-
		0.1	88, 93, 90, 88, 88	88 - 93 (n = 5)	89	2.2	-
		Overall		88 - 93 (n = 10)	91	2.9	-
Lettuce head	Ethofumesate-2-keto	Primary ion 256 m/z					
		0.01*	90, 91, 89, 88, 91	88 - 91 (n = 5)	90	1.5	-
		0.1	96, 98, 99, 97, 96	96 - 99 (n = 5)	97	1.3	-
		Overall		88 - 99	93	4.2	-

Matrix	Analyte	Fortification level (mg/kg)	Individual recoveries (%)	Range of recoveries (%) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
				(n = 10)			
		Confirmatory ion 177 m/z					
		0.01*	89, 92, 92, 90, 90	89 - 92 (n = 5)	91	1.5	-
		0.1	98, 100, 102, 99, 96	96 - 102 (n = 5)	99	2.1	-
		Overall		89 - 102 (n = 10)	91.5	6.1	-
		Confirmatory ion 121 m/z					
		0.01*	83, 78, 82, 86, 92	78 - 92 (n = 5)	84	6.1	-
		0.1	102, 94, 102, 99, 101	94 - 102 (n = 5)	100	3.4	-
		Overall		78 - 102 (n = 10)	92	9.8	-
Cereal grain	Ethofumesate-2-keto	Primary ion 256 m/z					
		0.01*	85, 88, 86, 93, 86	85 - 93 (n = 5)	88	3.3	-
		0.1	90, 91, 92, 92, 94	90 - 94 (n = 5)	92	1.6	-
		Overall		85 - 94 (n = 10)	90	3.3	-
		Confirmatory ion 177 m/z					
		0.01*	93, 93, 85, 95, 94	85 - 95 (n = 5)	90.0	3.5	-
		0.1	93, 91, 91, 92, 94	91 - 94 (n = 5)	93.1	7.9	-
		Overall		85- -94 (n = 10)	91.5	6.1	-
		Confirmatory ion 121 m/z					
		0.01*	89, 87, 77, 101, 101	77 – 101 (n = 5)	91	11.3	-
		0.1	94, 94, 94, 92, 96	92 – 96 (n = 5)	94	1.4	-
		Overall		77 – 101 (n = 10)	93	7.6	-

* 0.01 mg/kg = limit of quantification, defined by the lowest validated fortification level. Residues in duplicate control samples and reagent blanks were less than 30% of the LOQ.
**No Observable Peak
% Mean and % RSD calculated using rounded values.

Table A 2: Characteristics of the data collection analytical method used for the quantification of ethofumesate and ethofumesate-2-keto residues in lettuce and cereal grain

	Ethofumesate	**Ethofumesate-2-keto**
Selectivity	Final determination of ethofumesate and ethofumesate-2-keto was conducted by GC-MS monitoring 3 fragment ions per analyte; For each matrix a reagent blank and two control specimens were extracted and analysed to investigate the presence of ethofumesate / ethofumesate-2-keto and/or matrix interference at the retention time of ethofumesate / ethofumesate-2-keto. The selectivity of the method was demonstrated as no matrix interferences or residues of ethofumesate / ethofumesate-2-keto were observed at or above 30% of the LOQ in the reagent blank sample or the control samples. Representative chromatograms of a blank sample and control samples are presented in the report.	
Linearity/ calibration	The linearity of the detector was checked for each matrix by single injection of matrix-matched calibration standards at 6 concentration levels ranging from 0.0075 µg/mL (0.003 mg/kg) to 0.5 µg/mL (0.2 mg/kg). This calibration range is equivalent to 30% of the LOQ up to 200% of the upper fortification level. The calibration curves were linear with correlation coefficients (r) greater than 0.995. Detailed calibration data are presented in the report	
	Lettuce (286 m/z): y = 979014x + 5043.9, R = 0.9999 Lettuce (207 m/z): y = 2887905.5030x + 215.0197, R = 0.9999 Lettuce (161 m/z): y = 1795514.8434x + 13574.2348, R = 0.9999 Cereal grain (286 m/z): y = 679563x + 2660.5, R = 0.9996 Cereal grain (207 m/z): y = 2120950.6065x + 13546.4266, R = 0.9995 Cereal grain (161 m/z) y = 1382607.8686x + 11331.3604, R = 0.9996	Lettuce (256 m/z): y = 697917x + 308.97, R = 1.0000 Lettuce (177 m/z) y = 742185.2459x + 451.5430, R = 1.0000 Lettuce (121 m/z) y = 267102.5040x - 592.6808, R = 1.0000 Cereal grain (256 m/z) y = 513854x + 2107.9, R = 0.9998 Cereal grain (177 m/z) y = 533286.3852x + 1928.7410, R = 0.9997 Cereal grain (121 m/z) y = 177959.8951x + 1319.8996, R = 0.9990
Accuracy / Recovery	The accuracy and precision of the method was successfully demonstrated for each matrix type as the mean recovery values for ethofumesate and ethofumesate-2-keto at each fortification level were between 70 – 110% (see table above).	
Repeatability	The relative standard deviation (RSD) was ≤ 20% (see table above).	
Limit of quantification (LOQ)	The limit of quantitation (LOQ) was set to 0.01 mg/kg for all crops and each analyte, defined by the lowest validated fortification level.	
Limit of detection LOD	The limit of detection was confirmed to be less than 30 % of the LOQ for ethofumesate and ethofumesate-2-keto in each matrix as demonstrated by the response of the bottom calibration standard (equivalent to 30% of the LOQ) which was visually confirmed to be greater than three times signal to noise for all mass ions.	
Matrix effects	Matrix effects on detection were evaluated for ethofumesate / ethofumesate-2-keto were deemed to be significant (> 20 %) in some cases. Therefore, matrix-matched calibration standards were used for quantification.	
Extract Stability	Extract stability was assessed by re-injection of the LOQ recoveries using freshly prepared matrix matched calibration standards after at least 7 days refrigerated storage. Mean recovery values were in the acceptable range of 70 – 110% with an RSD of less than 20% and were within ± 20% of the original result. Final extracts were therefore shown to be stable when stored refrigerated for 7 days.	
Standard Stability	Solvent calibration standards were shown to be stable for 34 days when stored refrigerated which covers the period of time standards were used in the study. The peak area of a freshly prepared 0.025 µg/mL LOQ equivalent calibration standard (mean of three injections) was compared to the peak area of a stored standard of the same concentration (mean of three injections). The difference between the two was ≤ 10%.	

Conclusion

Analytical method RES-00278 has been successfully validated for the determination of residues of ethofumesate ethofumesate-2-keto in lettuce and cereal grain by GC-MS with a LOQ of 0.01 mg/kg.

Ecotoxicological analytical methods

Comments of zRMS:	Concentrations of ethofumesate were determined in tap water samples. Limit of Quantification: 30 µg/L. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate at LOQ=0.03 mg/L in tap water. The study is acceptable.

Reference:	KCP 5.1.2/02 (filed under KCP 10.2/01)
Report	Ethosat 500 Fish (*Golden Orfe*), Acute Toxicity Test, Semi-Static, 96 h, Scheerbaum D., 2005 Report no. FAG100321
Guideline(s):	SANCO/3029/99 rev. 4, ICH Harmonized Tripartie Guideline
Deviations:	Yes. Five recoveries at one fortification level instead of two fortification levels.
GLP:	Yes
Acceptability:	Yes. Validation meets guideline criteria (SANCO/3029/99 rev. 4, 11/07/2000) with minor deviations.

Materials and methods

The analytical method for ethofumesate determination was validated in tap water. The limit of quantification (LOQ) of the method was 0.03 mg/L. The quantitative measurements of ethofumesate were achieved by HPLC coupled to a UV detector.

Test substance:

Test substance	Ethosat 500
Batch No.	00401205
Active ingredient content	500 g ethofumesate/L (nominal), 508 g ethofumesate/L (actual)

Sample preparation

The samples were diluted 1:2 with mobile phase (acetonitrile + phosphoric acid (0.1%) (50:50; v/v)

Analytical method:

HPLC	Waters 712 WISP, UV-detector
Column	Nucleosil 100-5 C18 with pre-column, Nucleosil 100-5 C18
Column temperature	Room temperature
Detector	210 nm
Flow rate	0.7 mL/min
Mobile phase	Isocratic mode, 50 % acetonitrile + 50 % phosphoric acid (0.1 %)
Injection volume	100 µL
Retention time	Approx. 5.2 min

Results and discussions

Recovery results were in a range of 70 – 110% % with an RSD ≤ 20 per level. No outliers were identified. No interference (< 30 % LOQ) of total peak area for the target analytes were found in unfortified control samples. The LOQ was set at 0.03 mg/L.

Table A 3: Recovery results from method validation of ethofumesate in tap water

Matrix	Nominal concentration (mg a.i./L)	Analysed concentration (mg/L)	Recovery (%)	Mean recovery (%)	CV (%)
Tap water	0.0296	0.024 0.024 0.026 0.025 0.025	81 81 88 84 84	84	3.36

Table A 4: Characteristics for the analytical method used for validation of ethofumesate in tap water

	Ethofumesate
Specificity-interferences	Two dilution water blank samples were used to prove specificity and blank values being < 30 % of the LOQ.
Linearity/ calibration	The analytical system gave linear response in range of 0.5 - 16 mg/L of the standard. (Six levels prepared in mobile phase). The representative calibration curve and chromatograms are presented in the report. Correlation coefficients (R^2) were > 0.999.
Accuracy / Recovery	All recoveries were found to be between 70% and 110% for both the primary and confirmatory transitions in both matrices tested. (see table above).
Repeatability	RSD was below 20% (see table above).
LOQ	0.03 mg ethofumesate/L; corresponds to the lowest validated fortification level.
Matrix effects	Not relevant as samples and standard solutions were diluted with HPLC mobile phase

Conclusion

The analytical method fulfils the requirements of SANCO/3029/99 rev. 4 and is suitable for the determination of ethofumesate in tap water.

Comments of zRMS:	Concentrations of ethofumesate were determined in aqueous samples (ISO 6341standard water used for test OECD 202). Limit of Quantification: 0.7246 mg/L. Blank values do not exceed 30% of the lowest validated concentration. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate in test medium solution at LOQ=0.7246 mg/L. The study is acceptable.

Reference:	KCP 5.1.2/03 (filed under KCP 10.2/02)
Report	Renner P., 2020a: Acute toxicity of AG-E1-500 SC1 to *Daphnia magna* in a 48-hour static test, Report No: 20 48 ADL 0001
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for ethofumesate determination was validated in aqueous samples. The limit of quantification (LOQ) of the method for matrix charged water samples was 0.7246 µg/L. The quantitative measurements of ethofumesate were achieved by HPLC coupled to a MS/MS system (ethofumesate m/z 304.1→241.0).

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A
Active ingredient content	500 g ethofumesate/L (nominal), 529 g ethofumesate/L (actual)
Reference substance:	Ethofumesate
Batch No.	792174
Purity	99.7%

Sample preparation

The samples were thawed at room temperature and homogenized by shaking. Aliquots were diluted with validation diluent (methanol and test medium (50:50; v/v) in autosampler vials and measured by LC-M/MS.

Analytical method:

Method type	HPLC-MS
Equipment	Shimadzu LC-20ADXR pumps, Shimadzu DGU-20A3R degasser, Shimadzu SIL-20ACXR autosampler, Shimadzu CTO-20A column oven, Shimadzu CBM-20A controller, Shimadzu LabSolutions Version 5.86 data system
Column	ACE Excel 3 Super C18, 3 µm, 100 * 2.1 mm
Flow rate	0.4 mL/min
Mobile phase	A: 0.1% formic acid and 5 mM ammonium formate in water B: 0.1% formic acid and 5 mM ammonium formate in methanol 0.00 min 50% B 5.00 min 100% B 7.00 min 100% B 7.01 min 50% B Run time: 9.00 min
Retention time	Approx. 3.62 min
Detector	Shimadzu LCMS-8040, Triple quadrupole mass spectrometer
Detection	ESI positive, $[M+NH_4]^+$
Ions monitored	MRM m/z 304.1 → 241.0; m/z 304.1 → 121.1; m/z 304.1 → 161.1

Results and discussions

Recovery results were in a range of 70 – 110% % with an RSD ≤ 20 per level. No outliers were identified. No interference (< 30% LOQ) of total peak area for the target analytes were found in unfortified control samples. The LOQ was set at 0.7246 mg/L.

Table A 5: Recovery results from method validation of ethofumesate in ISO 6341 test medium (OECD 202)

Matrix	Nominal concentration (mg/L)	Dilution factor	Analysed concentration (mg/L)	Mean analysed concentration (mg/L)	Mean recovery (%)	RSD (%)
OECD 202 test medium	0.7246	20	0.680 0.673 0.699 0.681 0.703	0.6874	95	1.9
	23.53	20	22.94 22.47 22.61 20.49 19.36	21.57	92	7.3

Table A 6: Characteristics for the analytical method used for validation of ethofumesate in OECD 202 test medium (ISO 6341 Standard water)

	Ethofumesate
Specificity-interferences	The specificity was assured by MS/MS detection and the absence of interfering peaks. The blank and control values do not exceed 30% of the LOQ. Representative chromatograms of a blank sample and untreated test solution (control samples) was presented in the report.
Linearity/ calibration	The linearity of the HPLC-MS/MS detector response was determined by injecting seven calibration solutions in duplicate covering the range from 20% below the LOQ to 20% above the highest analyte concentration. The response was demonstrated to be linear over the concentration range of 28.46 µg/L to 569.2 µg/L. The representative calibration plots and equations have been provided. Correlation coefficients (R^2) were > 0.999. m/z = 304.1→ 121.0, Y=6268.5x + 33530.2, r^2 = 0.9999
Accuracy / Recovery	All recoveries were found to be between 70% and 110% for both the primary and confirmatory transitions in both matrices tested. (see table above).
Repeatability	RSD was below 20% (see table above).
LOQ	0.7246 mg/L; corresponds to the lowest validated level.
Matrix effects	Not relevant as samples and standard solutions were diluted with validation diluent (methanol/test medium; 50:50; v/v).

Conclusion

The analytical method fulfils the requirements of SANCO/3029/99 rev. 4 and is suitable for the determination of ethofumesate in ISO 6341standard water used for test OECD 202.

Comments of zRMS:	Concentrations of ethofumesate were determined in aqueous samples (OECD medium 201). Limit of Quantification: 0.959 mg/L. Blank values do not exceed 30% of the lowest validated concentration. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate in test medium solution at LOQ=0.959 mg/L. The study is acceptable.

Reference:	KCP 5.1.2/04 (filed under KCP 10.2/03)
Report	Renner P., 2020b: Effects of AG-E1-500 SC1 on *Desmodesmus subspicatus* in an algal growth inhibition test, Report No: 20 48 AAL 0001
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for ethofumesate determination was validated in aqueous samples (OECD medium 201). The limit of quantification (LOQ) of the method was 0.959 mg/L. The quantitative measurements of ethofumesate were achieved by HPLC coupled to a MS/MS system (ethofumesate m/z 304.1 → 241.0).

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A
Active ingredient content	500 g ethofumesate/L (nominal), 529 g ethofumesate/L (actual)
Reference substance:	Ethofumesate
Batch No.	792174
Purity	99.7%

Sample preparation

The samples were thawed at room temperature and homogenized by shaking. The samples were used as received without further dilution and analysed by LC-M/MS.

Analytical method:

Method type	HPLC-MS
Equipment	Shimadzu LC-20ADXR pumps, Shimadzu DGU-20A3R degasser, Shimadzu SIL-20ACXR autosampler, Shimadzu CTO-20A column oven, Shimadzu CBM-20A controller, Shimadzu LabSolutions Version 5.86 data system
Column	ACE Excel 3 Super C18, 3 µm, 100 * 2.1 mm
Injection volume	1 µL
Flow rate	0.4 mL/min
Mobile phase	A: 0.1% formic acid and 5 mM ammonium formate in water B: 0.1% formic acid and 5 mM ammonium formate in methanol 0.00 min 50% B 5.00 min 100% B 7.00 min 100% B 7.01 min 50% B Run time: 9.00 min
Retention time	Approx. 3.62 min
Detector	Shimadzu LCMS-8040, Triple quadrupole mass spectrometer
Detection	ESI positive, $[M+NH_4]^+$
Ions monitored	MRM m/z 304.1 → 241.0;

	m/z 304.1 → 121.1; m/z 304.1 → 161.1

Results and discussions

Recovery results were in a range of 70 – 110% % with an RSD ≤ 20 per level. No outliers were identified. No interference (< 30 % LOQ) of total peak area for the target analytes were found in unfortified control samples. The LOQ was set at 0.959 mg/L.

Table A 7: Recovery results from method validation of ethofumesate in OECD 201 test medium

Matrix	Nominal concentration (mg/L)	Dilution factor	Analysed concentration (mg/L)	Mean analysed concentration (mg/L)	Mean recovery (%)	RSD (%)
OECD 201 test medium	0.9586	2	1.015 0.9248 0.9514 0.9044 0.9085	0.9409	98	4.8
	4.993	2	5.167 5.193 5.255 5.175 5.163	5.190	105	0.7

Table A 8: Characteristics for the analytical method used for validation of ethofumesate in OECD 201 test medium

	Ethofumesate
Specificity-interferences	The specificity was assured by MS/MS detection and the absence of interfering peaks. The blank and control values do not exceed 30% of the LOQ. Representative chromatograms of a blank sample and untreated test solution (control samples) was presented in the report.
Linearity/ calibration	The linearity of the HPLC-MS/MS detector response was determined by injecting seven calibration solutions in duplicate covering the range from 20% below the LOQ to 20% above the highest analyte concentration. The response was demonstrated to be linear over the concentration range of 0.3828 to 3.063 mg/L. The representative calibration plots and equations have been provided. Correlation coefficients (R^2) were > 0.999. m/z = 304.1→ 121.0, Y= 3.15816e+006x+140631, r^2 = 0.9985
Accuracy / Recovery	All recoveries were found to be between 70% and 110% for both the primary and confirmatory transitions in both matrices tested. (see table above).
Repeatability	RSD was below 20% (see table above).
LOQ	0.959 mg/L; corresponds to the lowest validated level.
Matrix effects	Not relevant as samples and standard solutions were diluted with validation diluent (methanol/test medium; 50:50; v/v).

Conclusion

The analytical method fulfils the requirements of SANCO/3029/99 rev. 4 and is suitable for the determination of ethofumesate in OECD medium 201.

Comments of zRMS:	Concentrations of ethofumesate were determined in aquatic test medium (Smart and Barko medium). Limit of Quantification: 2.379 µg/L. Blank values do not exceed 30% of the lowest validated concentration. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate in test medium solution at LOQ=2.379 µg/L. The study is acceptable.

Reference:	KCP 5.1.2/05 (filed under KCP 10.2/04)
Report	Renner P., 2020c: Effects of AG-E1-500 SC1 on *Myriophyllum spicatum* in a static water sediment system Report No: 20 48 AMS 0001
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for ethofumesate determination was validated in aquatic test medium (Smart and Barko medium). The limit of quantification (LOQ) of the method was 2.379 µg/L. The quantitative measurements of ethofumesate were achieved by HPLC coupled to a MS/MS system (ethofumesate m/z 304.1 → 241.0).

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A
Active ingredient content	500 g ethofumesate/L (nominal), 529 g ethofumesate/L (actual)
Reference substance:	Ethofumesate
Batch No.	792174
Purity	99.7%

Sample preparation

The samples were thawed at room temperature and homogenized by shaking. Aliquots were diluted with validation diluent (methanol and test medium (50:50; v/v) in autosampler vials and measured by LC-M/MS.

Analytical method:

Method type	HPLC-MS
Equipment	Shimadzu LC-20ADXR pumps, Shimadzu DGU-20A3R degasser, Shimadzu SIL-20ACXR autosampler, Shimadzu CTO-20A column oven, Shimadzu CBM-20A controller, Shimadzu LabSolutions Version 5.86 data system
Column	ACE Excel 3 Super C18, 3 µm, 100 * 2.1 mm
Injection volume	1 µL
Flow rate	0.4 mL/min
Mobile phase	A: 0.1% formic acid and 5 mM ammonium formate in water B: 0.1% formic acid and 5 mM ammonium formate in methanol 0.00 min 50% B 5.00 min 100% B 7.00 min 100% B 7.01 min 50% B Run time: 9.00 min
Retention time	Approx. 3.62 min
Detector	Shimadzu LCMS-8040, Triple quadrupole mass spectrometer

Detection	ESI positive, $[M+NH_4]^+$
Ions monitored	MRM m/z 304.1 → 241.0; m/z 304.1 → 121.1; m/z 304.1 → 161.1

Results and discussions

Recovery results were in a range of 70 – 110% % with an RSD ≤ 20 per level. No outliers were identified. No interference (< 30 % LOQ) of total peak area for the target analytes were found in unfortified control samples. The LOQ was set at 0.2.379 µg/L.

Table A 9: Recovery results from method validation of ethofumesate in Smart and Barko test medium

Matrix	Nominal concentration (mg/L)	Dilution factor	Analysed concentration (mg/L)	Mean analysed concentration (mg/L)	Mean recovery (%)	RSD (%)
Smart and Barko test medium	2.379	2	2.473 2.427 2.597 2.704 2.629	2.566	108	4.4
	187.4	4	191.8 191.2 196.2 194.0 177.2	190.2	101	4.0

Table A 10: Characteristics for the analytical method used for validation of ethofumesate in Smart and Barko test medium

	Ethofumesate
Specificity-interferences	The specificity was assured by MS/MS detection and the absence of interfering peaks. The blank and control values do not exceed 30% of the LOQ. Representative chromatograms of a blank sample and untreated test solution (control samples) was presented in the report.
Linearity/ calibration	The linearity of the HPLC-MS/MS detector response was determined by injecting seven calibration solutions in duplicate covering the range from 20% below the LOQ to 20% above the highest analyte concentration. The response was demonstrated to be linear over the concentration range of 0.9148 to 57.1720 mg/L. The representative calibration plots and equations have been provided. Correlation coefficients (R2) were > 0.999. m/z = 304.1→ 121.0, Y= 71736.4x+8761.82, r^2 = 0.999
Accuracy / Recovery	All recoveries were found to be between 70% and 110% for both the primary and confirmatory transitions in both matrices tested. (see table above).
Repeatability	RSD was below 20% (see table above).
LOQ	2.379 µ/L; corresponds to the lowest validated level.
Matrix effects	Not relevant as samples and standard solutions were diluted with validation diluent (methanol/test medium; 50:50; v/v).

Conclusion

The analytical method fulfils the requirements of SANCO/3029/99 rev. 4 and is suitable for the determination of ethofumesate in Smart and Barko test medium.

Comments of zRMS:	Concentrations of ethofumesate were determined in larval diet (50% (w/v) aqueous sucrose solution). Quantification was performed by use of LC-MS/MS detection. Limit of Quantification: 374 mg test item/kg (171 mg ethofumesate/kg) with a limit of detection (LOD) set at 51.3 mg ethofumesate/kg (30% of the LOQ). Blank values do not exceed 30% of the lowest validated concentration. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate in aqueous sucrose solution at LOQ = 171 mg/kg. The study is acceptable.

Reference:	CP 5.1.2/06 (filed under KCP 10.3.1.2/01)
Report	Ansaloni T., 2020a:AG-E1-500 SC 1: Chronic Oral Toxicity Test (10-Day Feeding) to the Honey Bee, *Apis mellifera L*. under Laboratory Conditions, Report No: S19-20080, Sponsor Reference Number 000103264
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The QuEChERS analytical method for ethofumesate determination was validated in larval diet (50% aqueous sucrose solution). The limit of quantification (LOQ) of the method was 374 mg test item/kg (171 mg ethofumesate/kg). The quantitative measurements of ethofumesate were achieved by HPLC coupled to a MS/MS system (ethofumesate m/z 287 → 121).

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A
Active ingredient content	500 g ethofumesate/L (nominal), 511 g ethofumesate/L (analysed)
Reference substance:	Ethofumesate Pestanal analytical standard
Batch No.	BCBZ8303
Purity	99.5% area
Supplier	Sigma-Aldrich

Sample preparation - Sucrose Solution Recovery and Blank Samples:

For recovery and blank samples, 0.5 g ± 0.005 g of 50 % (w/v) aqueous sucrose solution (prepared at laboratory) were weighed accurately into 50 mL centrifuge tubes. Recovery samples were fortified at this step. 5 mL of water (HPLC grade) and 20 mL of acetonitrile were added to each tube and the samples were shaken. To each sample, one QuEChERS salt mixture (Bekolut Citrat-Kit-01) was added. The samples were shaken and centrifuged. The final sample extract was further diluted (dilution Factor DF 1) by a factor of 10 with acetonitrile/water (1:1,v/v) (= 100 µL of final sample extract + 900 µL of acetonitrile/water (1:1, v/v)). The samples were further diluted with matrix blank extract (dilution factor DF100 - 10000).

Sucrose Solution Matrix Blank Extract

Matrix blank extract for matrix calibration and dilution was prepared from blank samples from the respective sample preparation set. 5 mL of the acetonitrile extract after clean-up with "Bekolut Citrat-Kit-01" was mixed with 45 mL of acetonitrile/water (1:1, v/v) in a 50 mL plastic tube and shaken well.

Analytical method:

HPLC system	Thermo Accela 1250 HPLC pump with Thermo Accela Open autosampler
Pre-Column	HPLC guard column with 4 mm C18 cartridge (Phenomenex)
Column	Luna 5µ Phenyl-Hexyl., 150 mm x 2 mm, 5 µm (Phenomenex)
Injection volume	25 µL
Temperature	40 °C
Mobile phase	A: Water C: Methanol D: Water + 1 % formic acid

Gradient

Time	Eluent A	Eluent C	Eluent D	Flow rate (mL/min)
0.000	60	35	5	0.5
3.00	5	90	5	0.5
5.00	5	90	5	0.5
5.01	60	35	5	0.5
7.00	60	35	5	0.5

Retention time	Approx. 4.3 min
Mass spectrometric conditions	
MS system	Thermo TSQ Vantage triple quadrupole system
Ionisation type	Mass spectrometric conditions (MS/MS)
Polarity	ESI positive

Ions monitored

	Ion mass transition monitored [m/z]	Collision energy (V)	Quadrupole 1 Width [amu]	Quadrupole 3 width [amu]
Ethofumesate	m/z 287 → 121#	13	0.7	0.7
	m/z 287 → 77	47	0.7	0.7

used as qualifier

Results and discussions

Table A 11: Recovery results from method validation of ethofumesate in 50 %(w/v) aqueous sucrose solution

Matrix	Nominal concentration (mg/kg)	Ethofumesate concentration (mg/kg)	Recovery (%)	Mean recovery ± RSD (%)	Replicates	Overall mean recovery ± RSD (%)
Mass Transition m/z 287 → 121						
50 % (w/v) aqueous sucrose solution	Control		Ethofumesate not detectable (two replicates analysed)			
	374 (LOQ)	171	99, 98, 103, 90, 101	98 ± 5	5	96 ± 5
	18880	8615	89, 91, 93, 94, 100	93 ± 4	5	

RDS = relative standard deviation

Table A 12: Characteristics for the analytical method used for validation of ethofumesate in 50 % (w/v) aqueous sucrose solution

	Ethofumesate
Specificity-interferences	The analyte was determined in the final sample extracts by use of LC-MS/MS detection. One (1) mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated 50 % aqueous sucrose solution for accompanying control sample work up, for determination of recoveries and for preparation of matrix-matched calibration standards was available at the laboratory. Four control samples were analysed to investigate the residue level of the analyte and to check for any background interferences at the expected retention time of the analyte. The blank value at the expected retention time of the analyte of the control sample material that was used for determinations of the recoveries did not exceed 30 % of the LOQ. Since blank peaks were not observed, blank correction was not necessary. Example chromatograms representing control sample, the lowest calibration level, a sample fortified at the LOQ and a treated sample are included in report.
Linearity/	The linearity of the detector response was demonstrated by single determination of matrix-matched

	Ethofumesate
calibration	calibration standards for larval diet analysis at seven concentration levels ranging from 1 ng/mL to 10 ng/mL. This range covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any diluted sample (extract). The representative calibration plots and equations are presented in the report. m/z = 287 → 121, Y=-1133.67 + 16311x R^2 = 0.9973 W 1/X
Accuracy / Recovery	Five (5) recovery determinations at 374 mg test item/kg (171 mg ethofumesate/kg) (LOQ) and five (5) recovery determinations at 18880 mg test item/kg (8615 mg ethofumesate/kg) were performed. Two (2) control samples were analysed. (see table above). One mass transition was evaluated and representative ion chromatograms are shown in the report. A second mass transition was included to the LC-MS/MS method but used for monitoring only.
Repeatability	RSD was below 20% (see table above).
LOQ	374 mg test item/kg (171 mg ethofumesate/kg).
Matrix effects	Matrix effects were < ± 20 % and deemed to be insignificant. Nevertheless, matrix matched standards were used.

Conclusion
The method was successfully validated for determination of ethofumesate in 50% (w/v) aqueous sucrose solution with an LOQ of 374 mg test item/kg (171 mg ethofumesate/kg) and up to 18880 mg test item/kg (8615 mg ethofumesate/kg) according to the guidance document SANCO/3029/99, rev. 4.
With regard to selectivity, accuracy and precision, the analytical method was applied successfully when analysing the samples of the study.

Comments of zRMS:	Concentrations of ethofumesate were determined in larval diet samples. Quantification was performed by use of LC-MS/MS detection. Limit of Quantification: 53.4 mg test item/kg (24.4 mg ethofumesate/kg) with a limit of detection (LOD) set at 7.32 mg ethofumesate/kg (30% of the LOQ) for larval diet. Blank values do not exceed 30% of the lowest validated concentration. Recovery results were in a range of 70 – 110% with an RSD ≤ 20%. The validity criteria for the analytical method according to SANCO/3029/99 rev. 4 have been met. The method is suitable for the determination of ethofumesate in larva diet at LOQ of 24.4 mg/kg. The study is acceptable.

Reference:	CP 5.1.2/07 (filed under KCP 10.3.1.3/01)
Report	Ansaloni T., 2020b: AG-E1-500 SC 1: Honey Bee (*Apis mellifera L.*) Larval Toxicity Test following Repeated Exposure under laboratory conditions, Report No: S19-20081, Sponsor Reference Number 000103265
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The QuEChERS analytical method for ethofumesate determination was validated in larval diet. The limit of quantification (LOQ) of the method was 53.4 mg test item/kg (24.4 mg ethofumesate/kg). The quantitative measurements of ethofumesate were achieved by HPLC coupled to a MS/MS system (ethofumesate m/z 287 → 121).

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A

Active ingredient content	500 g ethofumesate/L (nominal), 511 g ethofumesate/L (analysed)
Reference substance:	Ethofumesate Pestanal analytical standard
Batch No.	BCBZ8303
Purity	99.5% area
Supplier	Sigma-Aldrich

Sample preparation - Larval Diet Recovery and Blank Samples:

For recovery and blank samples, 0.5 g of larval diet C were weighed into 50 mL centrifuge tubes. Recovery samples were fortified at this step. 5 mL water were added to each sample and the samples were shaken well. 20 mL acetonitrile were added to each tube and the tubes were shaken well. To each sample, one QuEChERS salt mixture (4 g magnesium sulfate + 1g sodium chloride + 1g trisodium citrate + 0.5 g disodium citrate sesquihydrate = Bekolut Citrat-Kit-01) was added and the samples were shaken well. The sample tubes were centrifuged for 2 minutes, at about 4000 rpm). The final sample extract was further diluted by a factor of 10 with acetonitrile/water (1:1, v/v) = 100 L of final sample extract + 900 µL of acetonitrile/water (1: 1, v/v). Further sample dilution with larval diet blank extract was performed to be within the calibration range. The samples were anlaysed by LC-M/MS.

Analytical method:

HPLC-System	Thermo Accela 1250 HPLC pump with Thermo Accela Open autosampler
Pre-Column	HPLC guard column with 4 mm C18 cartridge (Phenomenex)
Column	Luna 5µ Phenyl-Hexyl., 150 mm x 2 mm, 5 µm (Phenomenex)
Injection volume	25 µL
Temperature	40 °C
Mobile phase	A: Water C: Methanol D: Water + 1 % formic acid

Gradient

Time	Eluent A	Eluent C	Eluent D	Flow rate (mL/min)
0.000	60	35	5	0.5
3.00	5	90	5	0.5
5.00	5	90	5	0.5
5.01	60	35	5	0.5
7.00	60	35	5	0.5

Retention time	Approx. 4.2 min
Mass spectrometric conditions	
MS system	Thermo TSQ Vantage triple quadrupole system
Ionisation type	Mass spectrometric conditions (MS/MS)
Polarity	ESI positive

Ions monitored

	Ion mass transition monitored [m/z]	Collision energy (V)	Quadrupole 1 Width [amu]	Quadrupole 3 width [amu]
Ethofumesate	m/z 287 → 121#	13	0.7	0.7
	m/z 287 → 77	47	0.7	0.7

used as qualifier

Results and discussions

The mean recovery at each fortification level was in the range of 70- 110 % with a relative standard deviation of <20 % and thus comply with the standard acceptance criteria of the guidance document SANCO/3029/99 rev.4.

Table A 13: Recovery results from method validation of ethofumesate in larval diet

Matrix	Nominal concentration (mg/kg)	Ethofumesate concentration (mg/kg)	Recovery (%)	Mean recovery ± RSD (%)	Replicates	Overall mean recovery ± RSD (%)
Mass Transition m/z 287 → 121						
Larval diet C	Control		Ethofumesate not detectable (two replicates analysed)			
	53.5 (LOQ)	24.4	95, 112, 93, 94, 102	99 ± 8	5	98± 6
	3900	1780	93,97,99,98, 96	97 ± 2	5	

RDS = relative standard deviation

Table A 14: Characteristics for the analytical method used for validation of ethofumesate in larval diet

	Ethofumesate
Specificity-interferences	The analyte was determined in the final sample extracts by use of LC-MS/MS detection. One (1) mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated larval diet C for accompanying control sample work up, for determination of recoveries and for preparation of matrix-matched calibration standards were available at the laboratory. Two control samples were analysed to investigate the residue level of the analyte and to check for any background interferences at the expected retention time of the analyte. The blank value at the expected retention time of the analyte of the control sample material that was used for determinations of the recoveries did not exceed 30 % of the LOQ. Since blank peaks were not observed, blank correction was not necessary. Example chromatograms representing a larval diet control sample, the lowest calibration level, a sample fortified at the LOQ and a treated sample are included in report.
Linearity/ calibration	The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards for larval diet analysis at seven concentration levels ranging from 1 ng/mL to 10 ng/mL. This range covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any diluted sample (extract). The representative calibration plots and equations are presented in the report. m/z = 287 → 121, Y=-250.782 + 13580x R^2 = 0.9981 W 1/X
Accuracy / Recovery	All recoveries were found to be between 70% and 110% for both the primary and confirmatory transitions in both matrices tested. (see table above).
Repeatability	RSD was below 20% (see table above).
LOQ	53.4 mg test item/kg (24.4 mg ethofumesate/kg).
Matrix effects	Matrix effects on LC-MS/MS detection were investigated for larval diet and found to be insignificant. Nevertheless matrix-matched calibration standards were used for quantification of samples.

Conclusion

The method was successfully validated for determination of ethofumesate in larval diet C with a LOQ of 53.4 mg test item/kg and up to 3900 mg test item/kg according to the guidance document SANCO/3029/99, rev. 4.

With regard to selectivity, accuracy and precision, the analytical method was applied successfully when analysing the samples of the study. The results of dose verification show the correct dosage of test item to larval diet.

Comments of zRMS:	The method is suitable for the determination of ethofumesate in formulation quality control samples at LOQ=16 µg/mL. The study is acceptable.

Reference:	CP 5.1.2/08 (filed under KCP 10.6.2/01)
Report	Duffner A., 2020a: AG-E1-500 SC1: Effects on the Seedling Emergence and Seedling Growth of Non-Target Terrestrial Plant Species under Greenhouse Conditions, Report No: S19-22437, Sponsor Reference Number 000104143
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

And

Reference:	CP 5.1.2/09 (filed under KCP 10.6.2/02)
Report	Duffner A., 2020b: AG-E1-500 SC1: Effects on the Vegetative Vigour of Non-Target Terrestrial Plant Species under Greenhouse Conditions, Report No: S19-22438, Sponsor Reference Number 000104144
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was used for the rate verification of ethofumesate in formulation samples using LC-UV with detection at 228 nm.

Test substance:

Test substance	AG-E1-500 SC1
Batch No.	F4901-A
Active ingredient content	500 g ethofumesate/L (nominal), 529 g ethofumesate/L (analysed)
Reference substance:	Ethofumesate Pestanal analytical standard
Batch No.	BCBZ8303
Purity	99.5% area
Supplier	Sigma-Aldrich

Sample preparation:

All solutions were processed at room temperature allowing enough time to equilibrate. Analytical samples were diluted 1:10 in CH_3CN and 1:100 in solvent. All samples were analysed by HPLC-UV.

Analytical method:

HPLC-System	
Column	YMC-Pack ODS-A, 5 m (150 x 4.6 mm), YMC)
Injection, retention time	25 µL, 3 ±0.3 min
Time / Temperature / Flow	4.0 min at 60°C and 1.3 mL/min in isocratic mode
UV-Detection	228 nm

Results and discussions

Table A 15: Recovery results of ethofumesate in formulation quality control samples

Sample ID	Nominal concentration (µg a.i./mL	Calculated concentration (µg a.i./mL)		Replicates (n)	Mean	Accuracy %**
Date: of analysis, January 13, 2020						
QC 1	16.0	16.2	16.3	2	16.2	101.4
QC 2	28.8*	28.8**	29.2	2	29.0	100.7
QC 3	50.0	49.9	50.8	2	50.3	100.6
Date: of analysis, April 20, 2020						
QC 1	16.0	16.2	16.9	2	16.6	103.5
QC 2	28.8*	28.8**	29.9	2	29.5	102.3
QC 3	50.0	49.9	52.5	2	51.5	103.0

* round values
** based on unrounded values, considering the purity of the test item

Table A 16: Characteristics for the analytical method used for ethofumesate rate verification in formulation

	Ethofumesate
Specificity	The analyte was determined in the samples by use of LC-UV, a specific analytical method.
Linearity/ calibration	The range of calibration extended the expected concentrations of samples by ± 20 %. For validation a calibration curve with eight calibration standards in the range 10.0 to 100 µg/mL and quality controls with three concentration levels were used. The representative calibration plots and equations during sample measurement are presented in the report. Y=-349,696x – 81.181, r = 0.9998 1/X
Accuracy / Recovery	The precision of the method was reported as repeatability of recovery at each fortification level (LOQ and 10 x LOQ), as well as the overall relative standard deviation (RSD). At least five determinations were made at each fortification level. Quality control samples are presented in the report. (See table above).
Repeatability	No data
LOQ / LOD	LOD was determined to 0.048 µg ai/mL LOQ was calculated to 0.144 µg ai/mL using S/N>3

Conclusion

The measurement of the quality samples showed that the analytical method is fit for purpose for the determination of ethofumesate in aqueous samples.

A 2.1.2 Methods for post-authorization control and monitoring purposes for the active substance of ethofumesate (KCP 5.2)

In relation to residues in the commodities for which use of this product is proposed (sugar beet and fodder beet) and in appropriate animal matrices and environmental samples, the applicant has full access to the appropriate monitoring methods found acceptable in the DARs (or to studies which have been deemed equivalent) and the conclusions made on the basis of those studies. No further consideration is therefore necessary in this evaluation.

For validations of analytical methods used for data generation for risk assessment, please refer to A 2.1.1.