FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: GLOB2013F

Product name(s): Observer

Chemical active substance:

Zoxamide, 450 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: Globachem NV

Submission date: January 2024

Update: July 2024

MS Finalisation date: 19/12/2024

Version history

When	What
January 2024	Initial dossier submission by applicant for approval of new product
April 2024	Dossier sent for evaluation
July 2024	Applicant revision 01
September 2024	zRMS finalised evaluation
December 2024	zRMS finalised evaluation after commenting period

Table of Contents

zRMS comments:
This report has been completed by the Applicant.
The text highlighted in grey was provided by the zRMS.

5 Analytical methods

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are not available for the active substance(s) 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
Seed, ware and starch potato	Supported
Grape	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 zoxamide in plant protection product is provided as follows:

Comments of zRMS:	The method of analysis of active substance content (Zoxamide) in an SC Formulation containing 450 g/L Zoxamide has been validated in GLP laboratory in compliance with Document SANCO/3030/99 - rev 5. The acceptance criteria are met for all the validation parameters: - Linearity, - Sample Precision, - Recovery at 450 g/L. The species identification was confirmed by the MS and UV spectra. Specificity of the method – Zoxamide eluted at 11.0 minutes and there were no other peaks present at the same elution time as Zoxamide. The analytical method described in study report DNA6208: “Validation of the Methods of Determination of Zoxamide in an SC Formulation containing 450g/L Zoxamide, in compliance with Good Laboratory Practice” is applicable for the

	determination of the active ingredient Zoxamide in SC Formulation containing 450 g/L Zoxamide.

Reference:	KCP 5.1.1; Pomeroy, D.
Report	Validation of the Methods of Determination of Zoxamide in an SC Formulation containing 450g/L Zoxamide, in compliance with Good Laboratory Practice, DNA6208
Guideline(s):	SANCO 3030/99, rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	yes

Materials and methods

Zoxamide analysis

The assay of Zoxamide was performed using approximately 0.1g of sample. The mass of the Formulation was accurately recorded, transferred to a 100mL volumetric flask and partially made to volume with Acetonitrile. The sample was sonicated for 5 minutes, allowed to cool to room temperature, and made up to volume with Acetonitrile. Th samples were then assayed by injecting each solution once into the HPLC-PDA under the following conditions:

HPLC-PDA Conditions – Zoxamide Validation

Instrument:	Shimadzu HPLC-PDA
Mode:	Isocratic Reverse Phase
Column:	Grace Genesis C8 (250mm x 4.6mm)
Packing:	C8, 3µm
Eluent:	80% Acetonitrile 20% Deionised Water adjusted to pH3 with Formic Acid
Wavelength:	254nm
Flow Rate:	0.5 mL/minute
Injection Volume:	10µL
Column Temperature:	25ºC
Data Collection:	LabSolutions
Retention Time:	Approximately 10.9 to 11.0 minutes

ULTIVO LC-QQQ Conditions – Zoxamide MS Spectral Analysis

Instrument:	Agilent ULTIVO LC-QQQ Mass Spectrometer
Mode:	Isocratic Reverse Phase
Column:	Grace Genesis C8 (250mm x 4.6mm)
Packing:	C8, 3µm
Eluent:	80% Acetonitrile: 20% Deionised Water adjusted to pH3 with Formic Acid
Flow Rate:	0.5mL/minute
Injection Volume:	10µL
Column Temperature:	25ºC
Retention Time:	Approximately 10.7 minutes
Data Acquisition:	MassHunter

Ionisation:	Positive	Sheath Gas Temperature:	250°C
Gas Temperature:	150°C	Sheath Gas flow:	8L/minute
Gas Flow:	7L/minute	Capillary:	3500V
Nebulizer:	30psi	Nozzle Voltage:	2000V

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance zoxamide in plant protection product GLOB2013F

	Zoxamide
Author(s), year	Pomeroy, D.
Principle of method	HPLC-PDA
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity was determined from eighteen injections of nine concentrations of standard ranging from a blank to 1.0mg/mL. R^2= 0.9993
Precision – Repeatability Mean n = 6 (%RSD)	To show the sample precision, six samples of approximately 0.12g of sample (DNA6205/1) were prepared in 100mL Acetonitrile and injected into the HPLC-PDA. The values ranged from 446.3 g/L to 450.5g/L with a mean of 448.0g/L, a standard deviation of 1.714 and a percentage relative standard deviation of 0.383 y= 0.000000060x – 0.00557
Accuracy n = 6 (% Recovery)	The recovery samples were prepared for analysis by spiking the Blank formulation DNA3072/1 at 0.45mg/mL using the certified zoxamide reference standard. Six separate solutions were prepared and then injected into the HPLC-PDA. Recoveries ranged from 100.4% to 101.2% with mean of 101.0%, and a SD of 0.312. Recovery at 450g/L is between 97.5-103% Recovery at 0.5g/L (recovery range 100.4-101.2%) is between 75-125%
Interference/ Specificity	Specificity was determined by means of an analysis by HPLC-PDA. The zoxamide reference standard gave a peak at 10.9 minutes with a primary spectral maxima at 215nm, a secondary spectral maxima at 240nm, reducing to extrincrion byt 310nm. The sample DNA6205/1 gave a peak at 11.0 minutes with a primary spectral maxima at 215nm, a secondary spectral maxima at 240nm, reducing to extinction by 310nm in a similar manner to the zoxamdide reference standard, which confirms the specificity of the method. The specificity was further confirmed by means of an analysis by LC-QQQ. The zoxamide reference standard gave a peak at 10.7 minutes showing molecular ion of $[M+H]^+$ at 336m/z, with fragment ions present at 122.8m/z, 158.9 and 186.9m/z. The sample (DNA6205/1) gave a peak at 10.7 minutes showing the molecular ion of $[M+H]^+$ at 336m/z, with fragment ions present at 122.8m/z, 158.9m/z and 186.9m/z, in a similar manner to the reference standard which shows that the method is specific to Zoxamide. A procedure was developed that checks for interferences that may have

	Zoxamide
	occurred from other species that might mask the result of the expected analyte. In the Specificity chromatograms Zoxamide eluted at 11.0 minutes and other possible significant peaks were accounted for by assaying a solvent blank and the Blank Formulation (DNA6208/1). There were no other peaks present in these chromatograms at the same elution time as Zoxamide. This demonstrates that there were no analyte interferences and that the method is specific to Zoxamide.
Comment	

Conclusion

The validation parameters for the zoxamide methodology has been met for this study under SANCO/3030/99 – rev.5.

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

No relevant impurities are present in GLOB2013F.

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

Not required.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are no CIPAC methods available for the determination of zoxamide.

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 zoxamide and its metabolites RH-141452, RH-141455 and RH-150721 for the generation of pre-authorisation data is given in the following table. For the detailed evaluation of new/additional studies it is referred to Appendix 2.

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

Component of residue definition: zoxamide*				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Food/Feed of plant origin (Residues)	Primary	0.01 mg/kg	QuEChERS multiresidue method, LC-MS/MS	EFSA Journal 2017;15(9):4980

	Primary	0.01 mg/kg	QuEChERS multiresidue method, LC-MS/MS	Gustloff, C., 2022, S21-07039
	Primary	0.01 mg/kg	LC-MS/MS	Gustloff, C., 2022, S21-07040
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2023, S23-100692
	ILV	0.01 mg/kg	HPLC-MS/MS	Asekunowo, J., 2024, S23-100694
Food of animal origin (Residues)	Not triggered			EFSA Journal 2023;21:e8427
Soil (Environmental fate, Efficacy, Ecotoxicology)	Primary	0.05 mg/kg	LC-MS/MS	EFSA Journal 2017;15(9):4980
Drinking and surface water (Environmental fate, Efficacy, Ecotoxicology)	Primary	0.1 mg/kg	LC-MS/MS	EFSA Journal 2017;15(9):4980
Air (Exposure)	Primary	90 µg/m^3	LC-MS/MS	EFSA Journal 2017;15(9):4980
Feed, body fluids,... (Toxicology)	Primary	0.01 mg/kg	LC-MS/MS	Gustloff, C., 2023, S23-100691
Component of residue definition: RH-150721*				
Food/Feed of plant origin (Residues)	Primary	0.01 mg/kg (wine)	LC-MS/MS	Gustloff, C., 2022, S21-07042

* Plant residue definition for risk assessment: raw commodities: sum of zoxamide and RH-141452, expressed as zoxamide, processed commodities: RD-1: sum of zoxamide and RH-141452, expressed as zoxamide; RD-2 metabolite RH-150721

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

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

5.3.2 Description of analytical methods for the determination of residues of

zoxamide (KCP 5.2)

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 (apart from root crops where zoxamide is the monitoring residue definition now instead of RH-141452 and RH-141455 - EFSA Journal 2023;21:e8427).

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	Zoxamide	0.01 mg/kg 0.02 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Plant, high acid content		0.01 mg/kg 0.02 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg 0.02 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Plant, high oil content		0.01 mg/kg 0.02 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Plant, difficult matrices (hops, spices, tea)		0.01 mg/kg 0.05 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Honey		0.05 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Muscle	not triggered zoxamide for all products - MRL regulation	0.01 mg/kg	EFSA Journal 2023;21:e8427 Reg. (EU) 2017/171
Milk			
Eggs			
Fat			
Liver, kidney			
Soil (Ecotoxicology)	At least zoxamide but open regarding metabolites RH-163353 and RH-141455	0.05 mg/kg (zoxamide)	General limit for soil SANCO/825/00 rev. 8.1 EFSA Journal 2017;15(9):4980
Drinking water (Human toxicology)	At least zoxamide but open regarding RH -141455	0.1 mg/L	Meets general limit for drinking water SANCO/825/00 rev. 8.1
Surface water (Ecotoxicology)	At least zoxamide but open regarding RH-127450, RH-24549, RH-163353 & RH-141455	0.1 mg/L	Meets general limit for surface water SANCO/825/00 rev. 8.1
Air	zoxamide	90 µg/m^3	EFSA Journal 2017;15(9):4980
Tissue (meat or liver)	zoxamide	~~not required~~ 0.01 mg/kg	not classified as T SANTE/2020/12830 rev.2
Body fluids		~~not required~~ 0.01 mg/L	not classified as T SANTE/2020/12830 rev.2

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in plant matrices

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

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

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Potato (tuber, chips and flakes), grapes (berries, juice, wine and raisins), lettuce, dry bean and oilseed rape seed	Primary	0.01 mg/kg	QuEChERS multi-residue method, LC-MS/MS	EFSA Journal 2017;15(9):4980
	ILV (potato tuber, grape vine and lettuce)	0.01 mg/kg		EFSA Journal 2017;15(9):4980
	Primary	0.01 mg/kg	QuEChERS multiresidue method, LC-MS/MS	Gustloff, C., 2022, S21-07039
	Primary	0.01 mg/kg	LC-MS/MS	Gustloff, C., 2022, S21-07040
~~Honey~~	~~Primary~~	~~0.01 mg/kg~~	~~HPLC-MS/MS~~	~~Gustloff, C., 2023, S23-100692~~
	~~ILV~~	~~0.01 mg/kg~~	~~HPLC-MS/MS~~	~~Asekunowo, J., 2024, S23-100694~~

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
Required, available from:	EFSA Journal 2017;15(9):4980, Maric, A., 2023, S23-100483 Extraction efficiency was addressed in high water content commodities (pea whole plant) and dry commodities (dry peas) (Latvia, 2017).
Not required, because:	For zoxamide metabolites not required as no residues above LOQ are expected (cf. metabolism study)

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

An analytical method is not required due to the fact that no MRL is proposed.

zRMS comments:
zRMS does not agree with the Applicant's explanation. MRLs for zoxamide in products of animal origin were proposed by the Reg. (EU) No 2017/171 (0.01 mg/kg for all animal products, except honey for which the MRL value is 0.05 mg/kg). They are still valid.

According to the EFSA Journal 2017;15(9):4980: "An analytical method for food of animal origin is not proposed due to the fact that the residue definition for animals is currently open."

Taking into account that no residues are expected in potatoes or products of animal origin after use in accordance with the proposed GAP, the above mentioned lack of data is not considered critical for this dossier.

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

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2023, S23-100692
	ILV	0.01 mg/kg	HPLC-MS/MS	Asekunowo, J., 2024, S23-100694

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in soil is given in the following tables. ~~For the detailed evaluation of new/additional studies it is referred to Appendix 2.~~

Table 5.3-4: Validated methods for soil (if appropriate)

Component of residue definition: zoxamide			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.05 mg/kg	LC-MS/MS	EFSA Journal 2017;15(9):4980

~~For any special comments or remarkable points concerning the analytical methods for soil please refer to Appendix 2.~~

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in surface and drinking water is given in the following tables. ~~For the detailed valuation of new/additional studies it is referred to Appendix 2.~~

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

Component of residue definition: zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.1 µg/L	LC-MS/MS	EFSA Journal 2017;15(9):4980
	ILV	0.1 µg/L	LC-MS/MS	EFSA Journal 2017;15(9):4980
Surface water	Primary	0.1 µg/L	LC-MS/MS	EFSA Journal 2017;15(9):4980

~~For any special comments or remarkable points concerning the analytical methods for water please refer to Appendix 2.~~

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 zoxamide in air is given in the following tables. ~~For the detailed evaluation of new/additional studies please refer to Appendix 2.~~

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

Component of residue definition: zoxamide			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	90 µg/m^3	LC-MS/MS	EFSA Journal 2017;15(9):4980

~~For any special comments or remarkable points concerning the analytical methods for air it is referred to Appendix 2.~~

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

In the EFSA review on zoxamide (EFSA Journal 2017; 15(9):4980), a data gap was identified for an analytical method to monitor zoxamide in body fluids and tissues. Therefore, the applicant provides this method in the current dossier. An overview of this method is given in the following table. For the detailed evaluation of this new study, it is referred to Appendix 2.

Table 5.3-7: Methods for body fluids and tissues (if appropriate)

Component of residue definition: zoxamide and RH-141452				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Body fluids (urine) and animal tissues (liver)	Primary	0.01 mg/kg	LC-MS/MS	Gustloff, C., 2023, S23-100691

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

5.3.2.8 Other studies/ information

In several ecotoxicological studies summarized in section B9 of the dRR, analytical methods were used for the detection of zoxamide in the different test mediums. The analytical part of these studies is summarized in Appendix 2.

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	Pomeroy, D.	2021	Validation of the Methods of Determination of Zoxamide in an SC Formulation containing 450g/L zoxamide, in Compliance with Good Laboratory Practice, David Norris Analytical Laboratories Ltd, UK, Report No.: DNA6208, GLP, Unpublished	N	Globachem NV
KCP 5.1.1	Świstak, M.	2021	Validation of analytical method for the determination of active substance – zoxamide of the test item Zoxamide 450 SC in 50% sucrose solution, Sorbolab Research Laboratory Llc, Report No.: 0064/0014/FA, GLP, Unpublished	N	Globachem NV
KCP 5.1.1	Świstak, M.	2021	Validation of analytical method for the determination of active substance – zoxamide of the test item Zoxamide 450 SC in aqueous solutions, Sorbolab Research Laboratory Llc, Report No.: 0064/0011/FA, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Gustloff, C.	2022	Validation of Analytical Methods to Determine Residues of Zoxamide in Plant Matrices, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07039, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Gustloff, C.	2022	Validation of an Analytical Method to Determine Residues of Zoxamide Metabolites (RH-1452 and RH-1455) in Grape and Potato Matrices, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07040, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Gustloff, C.	2022	Validation of an Analytical Method for Determination of Zoxamide Metabolite RH-150721 in Wine, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07042, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Maric, A.	2023	Determination of Extraction Efficiency by Comparison of Methods for [14C]Zoxamide in Grape Plants, Eurofins Agroscience Services Ecochem Gmbh, Report No.: S23-100483, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	DeVellis, S.	2023	Zoxamide Metabolite (RH-163353) - Analytical Method Validation for the Determination of a Test Substance in Aqueous Solutions, Smithers Ers Ltd, Report No.: 14365.6100, GLP, Unpublished	N	Globachem NV

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
KCA 4.1.2	Liu, Y.	2023	RH-139432 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26104-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Liu, Y.	2023	RH-139432 in Algae Media Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26103-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Liu, Y.	2023	RH-127450 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26101-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Liu, Y.	2023	RH-127450 in Algae Media Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26102-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Liu, Y.	2023	RH-141455 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26105-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Liu, Y.	2023	RH-24549 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26107-22, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Gustloff, C.	2023	Validation of Analytical Methods for Determination of Propamocarb-HCl, Zoxamide and its metabolites RH-1452, RH-1455 and RH-150721 in Honey, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100692, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Gustloff, C.	2024	Validation of Analytical Methods for Determination of Propamocarb-HCl, Zoxamide and its metabolites RH-1452, RH-1455 and RH-150721 in Honey, Amendment 1 to Final Report, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100692, GLP, Unpublished	N	Globachem NV
KCA 4.1.2	Asekunowo, J.	2023	Independent Laboratory Validation of Analytical Methods for Determination of Propamocarb-HCl, Zoxamide and its Metabolites in Honey, Eurofins Agroscience Services Eag Laboratories Gmbh, Report No.: S23-100694, GLP, Unpublished	N	Globachem NV
KCA 4.2	Gustloff, C.	2023	Validation of an Analytical Method for Determination of Zoxamide in Body Fluids and Animal Tissues, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100691, GLP, Unpublished	N	Globachem NV

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 10.2.1 (filed in Part B Section 9)	Wilkins, S.	2023	GLOB2013F: *Daphnia magna* Acute Immobilisation Test, Fera Science Ltd, Report No.: FR/002721, GLP, Unpublished	N	Globachem NV
KCP 10.2.1 (filed in Part B Section 9)	Wright, E.	2023	GLOB2013F: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Fera Science Ltd, Report No.: FR/002720, GLP, Unpublished	N	Globachem NV
KCA 8.2.6.1 (filed in Part B Section 9)	Jarratt, N.	2023	Zoxamide Technical: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Fera Science Ltd, Report No.: FR/002786, GLP, Unpublished	N	Globachem NV
KCA 8.3.1.3 (filed in Part B Section 9)	Aguilar-Alberola, J.	2023	Zoxamide technical: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions, Eurofins Trialcamp S.L.U., Report No.: S23-106642, GLP, Unpublished	N	Globachem NV
KCP 10.6.2 (Submitted in Part B Section 9)	Dewson, S.	2023	GLOB2013F: OECD Terrestrial Plant Test - Vegetative Vigour Test, Report No.: STC/22/E1557, Laboratory: Stockbridge Technology Centre Ltd., GLP, Unpublished	N	Globachem NV
KCP 10.6.2 (Submitted in Part B Section 9)	Stead, A.	2023	GLOB2013F: OECD Terrestrial Plant Test - Seedling Emergence and Seedling Growth Test, Report No.: STC/22/E1558, Laboratory: Stockbridge Technology Centre Ltd., GLP, Unpublished	N	Globachem NV

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

*Studies owned by Globachem NV in the table below were generated to data match the AIR protected studies from the main notifier. The data matching package has been evaluated at EU level by the RMS Latvia and a copy was already sent to all MS.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCA 4.2	Homazava, N.	2022	Validation of LC-MS/MS Analytical Method for Zoxamide in Soil, Innovative Environmental Services, Report No.: 20210506, GLP, Unpublished	N	Globachem NV
KCA 4.2	Homazava, N.	2022	Validation of LC-MS/MS Analytical Method for Zoxamide in Water Matrices, Innovative Environmental Services, Report No.: 20210507, GLP, Unpublished	N	Globachem NV
KCA 4.2	Homazava, N.	2022	Validation of LC-MS/MS Analytical Method for Zoxamide in Air, Innovative Environmental Services, Report No.: 20210508, GLP, Unpublished	N	Globachem NV
KCP 4.2	Ducat, N.	2022	Determination of zoxamide residues in drinking water. Independent Laboratory Validation (ILV) of the analytical method described in the final report IES study 20210507 of Innovative Environmental Services (IES) Ltd, Switzerland for Globachem., Centre Wallon De Recherches Agronomiques, Report No.: 25674, GLP, Unpublished	N	Globachem NV

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for zoxamide

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

A 2.1.1.1 Description of analytical methods for the determination of residues in aqueous solutions (KCP 5.1)

A 2.1.1.1.1 Aquatic invertebrates

A 2.1.1.1.1.1 Method validation

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2.

Reference:	KCP 10.2.1
Report	GLOB2013F: *Daphnia magna* Acute Immobilisation Test, Wilkins, S, 2023, Fera Science Ltd, Report No.: FR/002721
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Apparatus
Electronic pipette e.g. Gilson Repetman
Glass pipettes
Volumetric flasks
HPLC amber vials

Materials
Zoxamide reference material
Daphnia media (M4 media), prepared on site
Elga pure water (Produced on site), or equivalent
Acetonitrile (ACN), HPLC grade or equivalent
Orthoposphoric acid (>85%), Fisher or equivalent

Preparation of matrix matched stock solution
Using an analytical balance and suitable glass volumetric flask, prepare a stock solution ca. 500 µg/mL of Zoxamide in ACN. Other concentrations may be used if appropriate.

Reagent Prep
HPLC mobile phase: 0.05% Aqueous H_3PO_4
Using a measuring cylinder, add 2L of Elga water to 2 litre glass media bottle. Using an electronic pipette (or equivalent), add 1.0 mL of orthophosphoric acid to the bottle. Cap and shake wll to mix. Degas in a sonication bath for at least 10 min. Other volumes may be used as long as the same concentration is maintained.

Results and discussions

Analytical Method Assessment – Zoxamide (Method FR/002721-A)

The analytical method was validated at 0.327 mg test item (TI)/L (low-level validation, LLV) and at 113 mg TI/L (high-level validation, HLV), equivalent to 0.1293 mg zoxamide/L and 44.812 mg zoxamide/L, respectively. The data from LIMS runs 65376 and 65410 show that the analytical method satisfies the validation guidelines in SANTE/2020/12830, Rev.2 as follows:

Matrix-Effects

An assessment of matrix effects on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix in run 65214.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

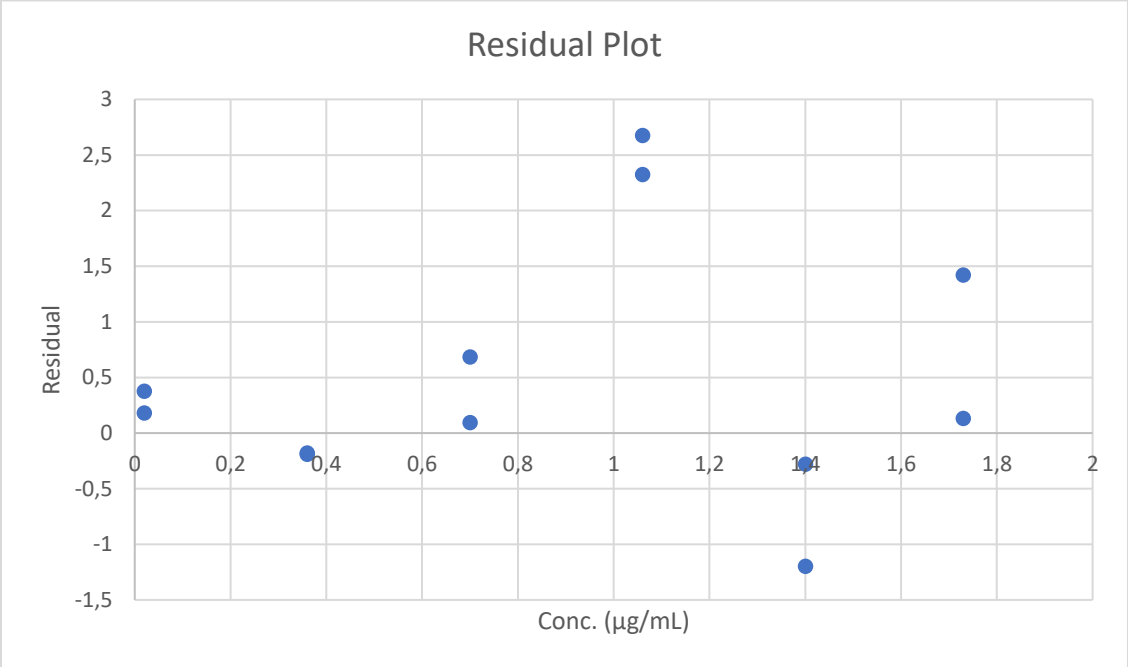
Matrix effects (%) = 100 x slope (matrix) / slope (solvent) - 100

For zoxamide, the matrix effect was determined as -0.35%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant. Hence solvents standards may be used.

Calibration

The calibration consisted of five levels with duplicate injections, bracketing the samples. The calibration range covered 2.00 to 0.02 µg zoxamide/mL. The LLV (= limit of quantification, LOQ) was performed at 0.1293 mg zoxamide/L (0.327 mg TI/L), which is equivalent to 0.06465 µg/mL when diluted according to method FR/002721-A. 30% of LOQ is 0.02 µg/mL, which is the lowest calibration level. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830, Rev.2 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. The correlation co-efficient (LIMS runs 65376), r, was 0.999994 (r^2 = 0.99999). The calibration graph met the principles of SANTE/2020/12830, Rev.2.

The calibration residuals were plotted and found to be randomly distributed.



A linear calibration function was therefore considered suitable for the quantitative determination of the target analyte. The calibration line was assessed for the LLV (LIMS Run 65376), all runs used the same cal line 0.02-2.0 µg/mL except the Matrix assessment (LIMS Run 65214) which was 0.03-3.0 µg/mL. This is within an order of magnitude, appear linear and has an r^2 value of 0.99997, therefore the assessment of the LLV cal line is suitable to cover the cal lines used in all runs.

Limit of detection

The lowest calibration level is 0.02 µg zoxamide/mL, equivalent to 0.04 mg zoxamide/L in sample.

Limit of quantification

This is equal to the lowest validation level which satisfies the SANTE/2020/12830, Rev.2 criteria, i.e. 0.1293 mg zoxamide/L (0.327 mg TI/L).

Recovery and repeatability

Two sets of fortified control samples were analysed, one set below the lowest expected sample concentration and one set above the highest expected sample concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

Run	Fortification level (mg TI/L)	Fortification level (mg zoxamide/L)	Recovery (%)	Mean recovery (%)	RSD (%)
LLV (**65376)**	0.0327	0.1293	102.11 104.90 103.71 105.65 103.80	104.03	1.29
HLV (65410)	113	44.812	99.59 99.30 99.62 99.85 99.56	99.59	0.20

For the method to be deemed fit for purpose, SANTE/2020/12830, Rev.2 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

Selectivity and specificity

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide, therefore, the method meets the requirements of SANTE/2020/12830, Rev.2 in this regard.

Extract Stability

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day or following a period of storage. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provides no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

Standard Stability

Stability of the analyte in the stored stock solution was determined in Fera study number FR/002722, where the stock zoxamide standard was shown to be stable in acetonitrile for at least 26 days when stored in a freezer.

Conclusion

Method FR/002721A v1 was assessed as being fit-for-purpose for this study.

A 2.1.1.1.1.2 Method validation RH-163353

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2.

Reference:	KCA 4.1.2
Report	Zoxamide Metabolite (RH-163353) - Analytical Method Validation for the Determination of a Test Substance in Aqueous Solutions, DeVellis, S., 2023, Smithers Ers Ltd, Report No.: 14365.6100
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an analytical method used to determine the content of RH-163353 in aqueous solutions. The method was validated (24 April to 26 May 2023) to quantify the concentrations of RH-163353 present in recovery samples prepared in Algal Assay Procedure (AAP) medium and dilute, natural, filtered seawater (FSW). The analytical method was validated with regards to selectivity and specificity, calibration, recovery and repeatability, limit of quantitation (LOQ), limit of detection (LOD), matrix effects, confirmation, and extract and stock stability in accordance with SANTE/2020/12830 rev. 2 (14 February 2023).

The method was validated in Algal Assay Procedure (AAP) medium and natural, dilute, filtered seawater (FSW) by fortification with RH-163353 at concentrations of 1.00 (LOQ) and 100,000 (High) µg/L. For each matrix, five replicate samples were produced for each concentration level. Additionally, two samples were left unfortified to serve as controls and one reagent blank was also prepared and processed with the fortified samples. Recovery samples were diluted with 20/80 acetonitrile/purified reagent water (v/v) for a final composition of 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). The High-level recovery samples were further diluted into the calibration range with 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Results and discussions

The method validation with RH-163353 in AAP medium met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	Confirmatory
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.	$r^2 = 0.994$	$r^2 = 0.991$
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	The matrix effects assessment indicated that there was no significant matrix effect observed (matrix effect <20%). This result is an indication that no significant matrix effect was observed. Even though no matrix effect was observed, there is a potential for matrix issues to become a concern during testing as the test vessels experience aging over the course of the exposure. As a result and as a conservative measure, matrix-matched calibration standards will be utilized for future testing with this method.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 120% for each fortification level will be considered acceptable.	LOQ, 1.00 µg/L: 98.2%	LOQ, 1.00 µg/L: 104%
		High, 100,000 µg/L: 98.3%	High, 100,000 µg/L: 98.0%
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 1.00 and 100,000 µg/L; 1.00 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 1.00 µg/L: 3.41%	LOQ, 1.00 µg/L: 5.30%
		High, 100,000 µg/L: 2.25%	High, 100,000 µg/L: 6.34%
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Final extract stability	Analyte stability in final extract will be sufficiently proven if recoveries of fortified samples are within 70.0 to 120% for each fortification level.	LOQ, 1.00 µg/L: 101 ± 1.50 % High, 100,000 µg/L: 113 ± 1.42 % This is an indication that the analyte was stable after 8 days of storage under refrigerated conditions (2 to 8 °C)	
Stock stability	Analyte stability in stock solution will be sufficiently proven if the means from at least 5 replicate measurements of a refrigerated aged and a freshly prepared stock solution do not differ by more than 10%	Primary Stock Solutions: 1000 mg/L: 2.77% Difference Secondary Stock Solutions: 10.0 mg/L: 5.18% Difference This is an indication that the analyte was stable after 37 days of storage under refrigerated conditions (2 to 8 °C).	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (1.00 µg/L).	All blank sample values were <30% of the LOQ 1.00 µg/L).
Limit Of Detection (LOD)	The LOD will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.300 µg/L	0.300 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 330.3/258.2 Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 330.3/159.0 Meets all method and guideline specifications outlined in this table.

The method validation with RH-163353 in FSW met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	Confirmatory
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.	$r^2 = 0.995$	$r^2 = 0.993$
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	The matrix effects assessment indicated that there was no significant matrix effect observed (matrix effect <20%). This result is an indication that no significant matrix effect was observed. Even though no matrix effect was observed, there is a potential for matrix issues to become a concern during testing as the test vessels experience aging over the course of the exposure. As a result and as a conservative measure, matrix-matched calibration standards will be utilized for future testing with this method.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 120% for each fortification level will be considered acceptable.	LOQ, 1.00 µg/L: 101%	LOQ, 1.00 µg/L: 97.0%
		High, 100,000 µg/L: 86.3%	High, 100,000 µg/L: 83.7%
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 1.00 and 100,000 µg/L; 1.00 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 1.00 µg/L: 5.76%	LOQ, 1.00 µg/L: 4.18%
		High, 100,000 µg/L: 4.71%	High, 100,000 µg/L: 2.28%
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Final extract stability	Analyte stability in final extract will be sufficiently proven if recoveries of fortified samples are within 70.0 to 120% for each fortification level.	LOQ, 1.00 µg/L: 104± 9.58 % High, 100,000 µg/L: 98.9 ± 5.59 % This is an indication that the analyte was stable after 8 days of storage under refrigerated conditions (2 to 8 °C)	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOD (1.00 µg/L).	All blank sample values were <30% of the LOQ 1.00 µg/L).
Limit Of Detection (LOD)	The LOD will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.300 µg/L	0.300 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 330.3/258.2 Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 330.3/159.0 Meets all method and guideline specifications outlined in this table.

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.2.

A 2.1.1.1.1.3 Method validation RH-127450

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-127450 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26101-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-127450, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methyl-N-(3-methyl-2-oxopentan-3-yl) benzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	99.93%
Linearity (Correlation Coefficient)	1.0000*
Precision (RSD from multiple injections of Check Standard)	0.45% (Check Standard C) 2.80% (Check Standard G)
Percent Recovery of Spiked Samples	102%
Limit of Detection	0.13 mg/L
Limit of Quantification	0.334 mg/L
Matrix Effect (<20%)	1.9849%
Blank Matrix Samples (at least 2)	Blanks values were 0.

* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.1.4 Method validation RH-24549

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-24549 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26107-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-24549, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-Dichloro-4-methylbenzoic acid (RH-24549), was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity, and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	99.56%
Linearity (Correlation Coefficient)	0.9999
Precision (RSD from multiple injections of Check Standard)	1.62% (Check Standard C) 2.27% (Check Standard F)
Percent Recovery of Spiked Samples	112%
Limit of Detection	0.32 mg/L
Limit of Quantification	1.159 mg/L
Matrix Effect (<20%)	3.6819%
Blank Matrix Samples (at least 2)	Blanks values were 0.

RSD - Relative Standard Deviation

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.1.5 Method validation RH-141455

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-141455 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26105-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-141455, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 2,6-Dichloroterephthalic acid (RH-141455), was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity, and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	101.43%
Linearity (Correlation Coefficient)	0.9998 (High Calibration Curve) 0.1000 (Low Calibration Curve)
Precision (RSD from multiple injections of Check Standard)	0.96% (Check Standard C) 2.47% (Check Standard G)
Percent Recovery of Spiked Samples	108% (Control) 70% (Low Spike) 104% (High Spike)
Limit of Detection	0.62 mg/L
Limit of Quantification	31.592 mg/L
Matrix Effect (<20%)	-1.5733%
Blank Matrix Samples (at least 2)	Blanks values were 0.

RSD - Relative Standard Deviation

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.1.6 Method validation RH-139432

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-139432 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26104-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-139432, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methylbenzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	100.96%
Linearity (Correlation Coefficient)	1.0000*
Precision (RSD from multiple injections of Check Standard C)	0.59% for active ingredient analysis 0.830% for storage stability analysis
Percent Recovery of Spiked Samples	99%
Limit of Detection	0.09 mg/L
Limit of Quantification	0.348 mg/L
Matrix Effect (<20%)	1.5434%
Blank Matrix Samples (at least 2)	Blanks values were 0.

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.2 Algae

A 2.1.1.1.2.1 Method validation

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2. Method FR/002722A v2 was assessed as being fit-for-purpose for this study.

Reference:	KCP 10.2.1
Report	GLOB2013F: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Wright, E., 2023, Fera Science Ltd, Report No.: FR/002720
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Apparatus
Electronic pipette e.g. Gilson Repetman
Glass pipettes
Volumetric flasks
HPLC amber vials

Materials
Zoxamide reference material
Elga pure water (Produced on site), or equivalent
Acetonitrile (ACN), HPLC grade or equivalent
Methanol, HPLC grade or equivalent (MeOH)
OECD algal media, supplied by aquatics team

Preparation of matrix matched stock solution
Using an analytical balance and suitable glass volumetric flask, prepare a stock solution ca. 250 µg/mL of Zoxamide in ACN. Other concentrations may be used if appropriate.

Reagent Prep
OECD algal media : MeOH (9:1, v/v)
Measure 450 mL OECD media with a measuring cylinder and transfer it to a suitable container. Measure 50 mL of MeOH with a measuring cylinder and add it to the container. Cap and swirl to mix. Allow mixture to cool to room temperature before using it. Other volumes may be used as long as the ratio is maintained and volumes recorded.
Water : MeOH (9:1, v/v)
Follow the method above with Elga water instead of OECD algal media. Other volumes may be used as long as the ratio is maintained and volumes recorded.

Results and discussions

Analytical Method Assessment – Determination of Zoxamide concentration in OECD algal media (Method FR/002786-B)

Product ion spectra were assessed as Fera GLP study LCMS Zoxamide-01 which demonstrated that the ions monitored were suitable.

The analytical method was validated at 0.005 mg zoxamide/L (low-level validation, LLV), and at 0.2 mg zoxamide/L (high-level validation, HLV). The data from LIMS run 65321 demonstrate that the analytical method satisfies the validation guidelines in SANTE/2020/12830 rev.2 as follows:

Matrix-Effects

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

Matrix effects (%) = 100 x mean peak area (matrix) / mean peak area (solvent) - 100

For zoxamide, the matrix effect was determined as -18.76%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant.

Clarification on Matrix assessment method:

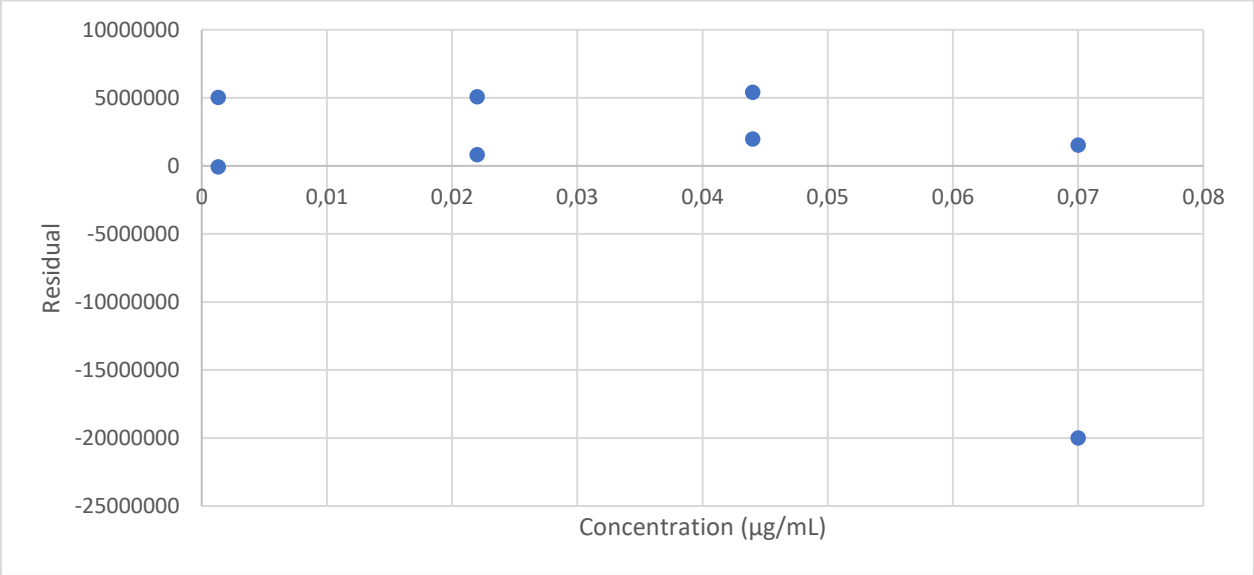
The method FR/002786-B was originally written for Fera Study FR/002786-B, the Test Item for which is technical grade Zoxamide. Due to the Test Item in this study (TI 1136 – GLOB2013F) being a formulated product, the matrix effect assessment used blank formulation and OECD media as the matrix. The method followed was:

For matrix matching, prepare a stock solution with the correct ratio of zoxamide (ref material) and blank formulant. TI 1136 has 448.0 g/L of Zoxamide and a density of 1.1346 g/mL. This is equivalent to 0.359 g/g of zoxamide/formulant.

Hence, to prepare a stock solution of 4 µg/mL of matrix matched solution of zoxamide, adjust the blank formulant concentration to 10 µg/mL. This can be done by preparing a 3600 µg/mL (other concentrations may be used if appropriate) solution of blank formulation in ACN and the Zoxamide stock solution in ACN as per analytical method. An assessment of the effect of blank formation and OECD algal media (sample matrix) on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

Calibration

The calibration curve consisted of four levels with duplicate injections, bracketing the samples. The calibration range covered 0.0013 – 0.07 µg zoxamide/mL. The LLV (= limit of quantification, LOQ) was performed at 0.005 mg zoxamide/L equivalent to 0.0045 µg/mL in extract when diluted according to method; 30% of this is 0.00135 µg zoxamide/mL. The lowest calibration level, 0.0013 µg/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. Although not required by Sante, the R^2 for the graph was 0.9701. The calibration graphs meet the principles of SANTE/2020/12830/Rev.2.



Limit of detection

The lowest calibration level is 0.0013 µg zoxamide/mL, equivalent to 0.00143 mg zoxamide/L in undiluted sample based on dilution scheme followed for the controls and LLV.

Limit of quantification

This is equal to the lowest validation level which satisfies the SANTE/2020/12830/Rev.2 criteria, i.e. 0.005 mg zoxamide/L.

Selectivity and specificity

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide (~6.2 minutes), therefore, the method meets the requirements of SANTE/2020/12830/Rev.2 in this regard.

Recovery and repeatability

Three sets of fortified control samples were analysed, one set below the lowest expected sample concentration, one set above the highest expected sample concentration and one set at an intermediate concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

Fortification level (mg zoxamide/L)	Recovery (%)	Mean recovery (%)	RSD (%)
0.005	81.61 79.37 76.40 77.60 74.93	77.98	3.33
0.2	88.22 83.18 83.92 85.97 83.18	84.89	2.57

For the method to be deemed fit for purpose, SANTE/2020/12830 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

Extract Stability

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

Standard Stability

Stability of the analyte in the stored stock solution was assessed (LIMS run 65362) by comparing the response of a calibration standard prepared from a freshly prepared stock with a calibration standard freshly prepared from a stock solution stored for 21 days. The mean peak area of the older standard was 10.59% lower than the mean peak area of the fresh standard. As the responses were not within 10% of each other, the stock zoxamide standard has been shown to not be stable in acetonitrile for 21 days when stored in a freezer. Fresh stocks should be prepared for each analytical run or a shorter storage period should be assessed.

Conclusion

Method FR/002786-B was assessed as being fit-for-purpose for this study.

A 2.1.1.1.2.2 Method validation zoxamide technical

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2. Method FR/002786-B was assessed as being fit-for-purpose for this study.

Reference:	KCP 10.2.1
Report	Zoxamide Technical: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Jarratt, N., 2023, Fera Science Ltd, Report No.: FR/002786
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for the determination of Zoxamide concentration in OECD algal media was validated as part of Fera Study FR/002720.

Apparatus
Electronic pipette e.g. Gilson Repetman
Glass pipettes
Volumetric flasks
HPLC amber vials

Materials

Zoxamide reference material
Elga pure water (Produced on site), or equivalent
Acetonitrile (ACN), HPLC grade or equivalent
Methanol, HPLC grade or equivalent (MeOH)
OECD algal media, supplied by aquatics team

Preparation of matrix matched stock solution
Using an analytical balance and suitable glass volumetric flask, prepare a stock solution ca. 250 µg/mL of Zoxamide in ACN. Other concentrations may be used if appropriate.

Reagent Prep
OECD algal media : MeOH (9:1, v/v)
Measure 450 mL OECD media with a measuring cylinder and transfer it to a suitable container. Measure 50 mL of MeOH with a measuring cylinder and add it to the container. Cap and swirl to mix. Allow mixture to cool to room temperature before using it. Other volumes may be used as long as the ratio is maintained and volumes recorded.
Water : MeOH (9:1, v/v)
Follow the method above with Elga water instead of OECD algal media. Other volumes may be used as long as the ratio is maintained and volumes recorded.

Results and discussions

Method FR/002786-B was validated at 5 µg zoxamide/L (low-level validation, LLV), and at 200 µg zoxamide/L (high-level validation, HLV).

The data from LIMS run 65321 demonstrate that the analytical method satisfies the validation guidelines in SANTE/2020/12830 rev.2 as follows:

Matrix-Effects

An assessment of the effect of blank formation and OECD algal media (sample matrix) on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

Matrix effects (%) = 100 x mean peak area (matrix) / mean peak area (solvent) - 100

For zoxamide, the matrix effect was determined as -18.76%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant.

Study FR/002786 used technical grade zoxamide (no formulation). A matrix assessment of just OECD algal media was not carried out, however as no significant matrix effect was seen for blank formation and OECD media, it can be assumed the media on its own would not cause any significant matrix effects.

Calibration

The calibration curve consisted of four levels with duplicate injections, bracketing the samples. The calibration range covered 0.0013 – 0.07 µg zoxamide/mL. The LLV (= limit of quantification, LOQ) was performed at 5 µg zoxamide/L equivalent to 0.0045 µg/mL in extract when diluted according to method;

30% of this is 0.00135 µg zoxamide/mL. The lowest calibration level, 0.0013 µg/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. Although not required by Sante, the R^2 for the graph was 0.9701. The calibration graphs meet the principles of SANTE/2020/12830/Rev.2.

Limit of detection

The lowest calibration level is 0.0013 µg zoxamide/mL, equivalent to 1.43 µg zoxamide/L in undiluted sample based on dilution scheme followed for the controls and LLV.

Limit of quantification

This is equal to the lowest validation level which satisfies the SANTE/2020/12830/Rev.2 criteria, i.e. 5 µg zoxamide/L.

Selectivity and specificity

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide, therefore, the method meets the requirements of SANTE/2020/12830/Rev.2 in this regard.

Recovery and repeatability

Three sets of fortified control samples were analysed, one set below the lowest expected sample concentration, one set above the highest expected sample concentration and one set at an intermediate concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

Fortification level (µg zoxamide/mL)	Recovery (%)	Mean recovery (%)	RSD (%)
5	81.61 79.37 76.40 77.60 74.93	77.98	3.33
200	88.22 83.18 83.92 85.97 83.18	84.89	2.57

For the method to be deemed fit for purpose, SANTE/2020/12830 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

Extract Stability

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

Standard Stability

Stability of the analyte in the stored stock solution was assessed (LIMS run 65362) by comparing the response of a calibration standard prepared from a freshly prepared stock with a calibration standard freshly prepared from a stock solution stored for 21 days. The mean peak area of the older standard was 10.59% lower than the mean peak area of the fresh standard. As the responses were not within 10% of each other, the stock zoxamide standard has been shown to not be stable in acetonitrile for 21 days when stored in a freezer. Fresh stocks should be prepared for each analytical run or a shorter storage period should be assessed.

Conclusion

Method FR/002786-B was assessed as being fit-for-purpose for this study.

A 2.1.1.1.2.3 Method validation RH-163353

Please refer to A 2.1.1.1.2.2.

A 2.1.1.1.2.4 Method validation RH-127450

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-127450 in Algae Media Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26102-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-127450 in Algae Media, within an algae media matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard and ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methyl-N-(3-methyl-2-oxopentan-3-yl) benzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects,

linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	100.12%
Linearity (Correlation Coefficient)	1.0000*
Precision (RSD from multiple injections of Check Standard)	0.44% (Check Standard C) 3.73% (Check Standard G)
Percent Recovery of Spiked Samples	108%
Limit of Detection	0.13 mg/L
Limit of Quantification	0.370 mg/L
Matrix Effect (<20%)	1.4020%
Blank Matrix Samples (at least 2)	Blanks values were 0.

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve.

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.2.5 Method validation RH-139432

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCA 4.1.2
Report	RH-139432 in Algae Media Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26103-22
Guideline(s):	OCSPP 830.1800 SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-139432 in Algae Media, within an algae media matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard and ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methylbenzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity,

Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

Results and discussions

Method Validation	
Specificity and Selectivity	Confirmation of identity by UV/Visible Spectra retention time and absorbance
Average Accuracy of Standards	100.96%
Linearity (Correlation Coefficient)	1.0000*
Precision (RSD from multiple injections of Check Standard C)	0.59% for active ingredient analysis 0.788% for storage stability analysis
Percent Recovery of Spiked Samples	102%
Limit of Detection	0.09 mg/L
Limit of Quantification	0.315 mg/L
Matrix Effect (<20%)	0.3215%
Blank Matrix Samples (at least 2)	Blanks values were 0.

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve.

Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

A 2.1.1.1.3 Bees

A 2.1.1.1.3.1 Method validation in aqueous solutions

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCP 5.1.1
Report	Validation of analytical method for the determination of active substance – zoxamide of the test item Zoxamide 450 SC in aqueous solutions, Świstak, M., 2021, SORBOLAB Research Laboratory LLC, Report No.: 0064/0011/FA
Guideline(s):	SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Validation of analytical method was based on experimental procedure SPB-FA/11 and Guideline SANTE/2020/12830, rev.1.

During the validation of the analytical method the following parameters: selectivity, matrix effects, linearity, accuracy, precision (repeatability), limit of detection and limit of quantification were determined. Determination of active substance (zoxamide) in aqueous solution of the test item was performed by high performance liquid chromatography with UV-DAD detection on the basis of signal from active substance. Identification of active substance was made by comparing the UV spectrums and retention times of standard solutions and solutions of the test item.

Reagents and solutions
- zoxamide standard, dr Ehrenstorfer, lot number G1038119 (quality certificate Appendix 2)
- acetonitrile, HPLC grade, POCH, lot number 1251/02/21
- methanol, HPLC grade, Avantor, lot number 1225/07/20
- orthophosphoric acid 85% p.a, Chempur, lot number 19/04/08
- Triton X-100, Chempur, lot number 210616118
- deionized water
- ultrapure water
- 0.1% (v/v) orthophosphoric acid (prepared by adding 1.176 mL of orthophosphoric acid 85%, p.a. to 1000 mL volumetric flask filled with 900 mL of ultrapure water, and then filling to the mark with ultrapure water)
- 0.1% (w/v) Triton X- 100 solution in deionized water (prepared by weighing 0.1 g of triton X-100 to 100 mL volumetric flask and then filling up to the mark with deionized water)

Equipment
- high performance liquid chromatography Shimadzu Nexera series LC-30 with PDA detector
- analytical balance Radwag XA 82_220.4Y.A
- precision balance Ohaus series PA 2102CM-1
- adjustable automatic pipettes: Transferpette S 10 µL, Transferpette S 1 mL
- deionizer SolPure 78
- system for obtaining ultrapure water Millipore Synergy UV
- ultrasonic bath Sonic-10
- volumetric flask class A
- syringes and syringe filters 0.22 µm

Chromatographic conditions

Column	Kinetex 2.6 µm F5 100A 100x4.60 mm
Detection	235 nm
Injection volume	10 µL
Column thermostat temperature	40°C
Mobile phase	Acetonitrile : 0.1% H3PO4 (70:30)
Flow of mobile phase	0.8 mL/min

Preparation of standard solution
25.30 mg of zoxamide standard was weighed into a 10 mL graduated flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. The zoxamide standard solution with concentration of 2481.424 mg/L (purity of standard 98.08%) was obtained. The prepared solution was diluted with methanol and filtrated through syringe filters.

Preparation of test item solution
50.64 mg of test item was weighed into a 10 mL graduated flask. The flask was filled up to the mark with Triton X-100 solution and whole was mixed thoroughly. Test item solution in Triton X-100 solution with concentration of 5064 mg/L was obtained. Separately, 49.70 mg of test item was weighed into a 10 mL graduated flask. The flask was filled up to the mark with deionized water and whole was mixed thoroughly. Test item solution in deionized water with concentration of 4970 mg/L was obtained.The solutions prepared in this way were diluted with methanol and filtrated through syringe filters.

Results and discussions

Validation method was based on experimental procedure SPB-FA/11 and guideline SANTE/2020/12830, rev.1.

Validation parameters for method are shown in the following table.

Parameter	Required criterion	The result			
Selectivity	at the place originating of active substance signal, there is no signals originating of other substances of area exceeding 30% of active substance area in the test item solution at the level (LOQ) the UV spectrum of active substance in the standard solution and the test item solution is comparable	at the place originating of active substance signal, there is no signals originating of other substances of area exceeding 30% of active substance area in the test item solution at the level (LOQ) the UV spectrum of active substance in the standard solution and the test item solution is comparable			
Linearity	r ≥ 0.99 random distribution of regression residuals	r = 0.999 (0.22333 mg/L – 10.17384 mg/L) random distribution of regression residues was obtained			
Matrix effect [%]	±20	Test item solution in 0.1% (w/v) Triton X-100 solution	-2.32		
		Test item solution in deionized water	-3.44		
Accuracy [%]	70-120	Test item solution in 0.1% (w/v) Triton X-100 solution	level I	98.4	99
			level II	100.5	
		Test itemsolution in deionized water	level I	105.4	102
			level II	98.6	
Precision [% RSD]	≤ 20	Test item solution in 0.1% (w/v) Triton X-100 solution	level I	1.67	
			level II	2.13	
		Test itemsolution in deionized water	level I	0.56	
			level II	0.50	
Limit of detection [mg/L]	≤ 0.29436 mg/L (for test item in 0.1% (w/v) Triton X-100 solution) ≤ 0.31534 mg/L (for test item solution in deionized water)	0.22333 mg/L			
Limit of quantification [mg/L]	-	0.98119 mg/L (for test item solution in 0.1% (w/v) Triton X-100 solution) 1.05113 mg/L (for test item solution in deionized water)			

Conclusion

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance (zoxamide) in aqueous solution of the test item Zoxamide 450 SC.

A 2.1.1.1.3.2 Method validation in 50% sucrose solution

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 1.

Reference:	KCP 5.1.1
Report	Validation of analytical method for the determination of active substance - zoxamide of the test item Zoxamide 450 SC in 50% sucrose solution, Świstak, M., 2021, SORBOLAB Research Laboratory LLC, Report No.: 0064/0014/FA
Guideline(s):	SANTE/2020/12830, rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Test method
Validation of analytical method was based on experimental procedure SPB-FA/11 and Guideline SANTE/2020/12830, rev.1.
During the validation of the analytical method the following parameters: selectivity, matrix effects, linearity, accuracy, precision (repeatability), limit of detection and limit of quantification were determined. Determination of active substance (zoxamide) of the test item in 50% sucrose solution was performed by high performance liquid chromatography with UV-DAD detection on the basis of signal from active substance. Identification of active substance was made by comparing the UV spectrums and retention times of standard solution and test item solution.

Reagents and solutions
- acetonitrile, HPLC grade, POCH, lot number 1251/02/21
- methanol, HPLC grade, Avantor, lot number 1225/07/20
- sodium chloride, p.a, Chempur, lot number 210409251
- ethyl acetate, HPLC grade, Avantor, lot number 0850/10/18
- orthophosphoric acid 85% p.a, Chempur, lot number 19/04/08
- 50% sucrose solution provided by the ecotoxicology laboratory
- 0.1% (v/v) orthophosphoric acid (prepared by adding 1.176 mL of orthophosphoric acid 85%, p.a. to 1000 mL volumetric flask filled with 900 mL of ultrapure water, and then filling up to the mark with ultrapure water)
- deionized water
- ultrapure water
- zoxamide standard, dr Ehrenstorfer, lot number G1038119 (quality certificate Appendix 2)

Equipment
- high performance liquid chromatography Shimadzu Nexera series LC-30 with PDA detector
- analytical balance Radwag XA 82_220.4Y.A
- precision balance Ohaus series PA 2102CM-1
- adjustable automatic pipettes: Transferpette S 1 mL, Transferpette S 5 mL
- roller shaker RM10V-W
- apparatus for concentration of samples NDK200
- vacuum pomp Basic20 PL 2/2 type

- deionizer SolPure 78
- system for obtaining ultrapure water Millipore Synergy UV
- ultrasonic bath Sonic-10
- stopwatch ISOLAB
- volumetric flask class A
- syringes and syringe filters 0.22 µm

Chromatographic conditions
Column Kinetex 2.6 µm F5 100A 100x4.60 mm
Detection 254 nm
Injection volume 25 µL
Column thermostat temperature 40°C
Mobile phase Acetonitrile : 0.1% H3PO4 (70 : 30)
Flow of mobile phase 0.8 mL/min

Preparation of standard solution
25.30 mg of zoxamide standard was weighed into a 10 mL volumetric flask. The flask was filled up to the mark with methanol and whole was mixed thoroughly. The zoxamide standard solution with concentration of 2481.424 mg/L (purity of standard 98.08%) was obtained. 0.5 mL of the prepared solution was diluted with 50% sucrose solution to a weight of 20.00413 g.
Concentration of zoxamide in 50% sucrose solution was calculated according to the formula:

$$C_{AS} = \frac{C_{TI} * C\%}{100\%}$$

where:

C_{AS}	concentration of zoxamide in 50% sucrose solution [mg/kg]
C_{TI}	concentration of test item in 50% sucrose solution [mg/kg]
C%	concentration of zoxamide in the test item [% (w/w)]

The prepared solution of test item in 50% sucrose solution was diluted with 50% sucrose solution and the test item solutions were prepared as follows.

Preparation of sample for analysis
5 g of sample was weighed into a tube with an accuracy of 0.01 mg. 0.5 g of sodium chloride and 2 mL of ethyl acetate was added and whole was shaken for 45 minutes on the roller shaker. Next, 1 mL of ethyl acetate was transferred into a Eppendorf tube. The solvent was evaporated and 1 mL of methanol was added to residue. Whole was shaken thoroughly and filtered through a syringe filter into a chromatography vial.
Concentration of zoxamide in prepared sample was calculated according to the formula:

$$C_{mg/L} = \frac{C_{mg/kg} * m_t}{2}$$

where:

$C_{mg/L}$	zoxamide concentration in prepared sample [mg/L]
$C_{mg/kg}$	zoxamide concentration in 50% sucrose solution [mg/kg]
m_t	mass of sample taken for extraction [g]
2	value resulting from sample preparation

Results and discussions

Validation of analytical method for determination of active substance (zoxamide) of the test item Zoxamide

450 SC in 50% sucrose solution was conducted. During the validation of the analytical method the following parameters: selectivity, matrix effects, linearity, accuracy, precision (repeatability), limit of detection and limit of quantification were determined.
Validation method was based on experimental procedure SPB-FA/11 and guideline SANTE/2020/12830, rev.1.
Validation parameters for method are shown in the following table.

Parameter	Required criterion	The result		
Selectivity	at the place originating of active substance signal, there is no signals originating of other substances of area exceeding 30% of active substance area in the test item solution at the level (LOQ) the UV spectrum of active substance in the standard solution and the test item solution is comparable	at the place originating of active substance signal, there is no signals originating of other substances of area exceeding 30% of active substance area in the test item solution at the level (LOQ) the UV spectrum of active substance in the standard solution and the test item solution is comparable		
Linearity	r ≥ 0.99 random distribution of regression residuals	r = 0.999 (0.58632 mg/L – 25.51686 mg/L) random distribution of regression residues was obtained		
Matrix effect [%]	±20	9.70		
Accuracy [%]	70-120	level I	102.9	104
		level II	104.6	
Precision [% RSD]	≤ 20	level I	0.95	
		level II	3.11	
Limit of detection [mg/L]	≤ 0.90474	0.58632		
Limit of quantification [mg/L]	-	3.01580		

Conclusion

Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance (zoxamide) of the test Zoxamide 450 SC in 50% sucrose solution.

A 2.1.1.1.3.3 Bees larvae chronic - method validation - zoxamide technical

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2.

Reference:	KCA 8.3.1.3
Report	Zoxamide technical: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions, Aguilar-Alberola, J., 2023, Eurofins Trialcamp S.L.U., Report No.: S23-106642
Guideline(s):	SANTE/2020/12830, rev. 1

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, sample extraction of larval diet containing 1.5 % acetone diet were performed by dilution of samples with acetonitrile/water (1:1; v/v). Quantification was performed by use of LC-MS/MS.

Results and discussions

The limit of quantification (LOQ) was 2.97 mg zoxamide /kg with a limit of detection (LOD) of 0.619 mg/kg.
The maximum storage interval from sampling until extraction was 23 days.
The storage temperature of the samples at the analytical test site was ≤ -18 °C with no exceedance.
Storage stability testing was not necessary because the interval from sampling until injection did not exceed 30 days for any analysed sample.
Results in mg/kg are reported without correction for the obtained concurrent recoveries, i.e. no adjustments to hypothetical concurrent recoveries of 100 % were made.
The following concentrations were detected in the samples:

Sample Name	*Active Ingredient Nominal (mg/kg)	Calculated Concentration of Active Ingredient (mg/kg)	Recovery (%)	Mean Recovery (%)
S23-106642-L2-D3-T1-A	32.466	28.004	86.3	95.6
S23-106642-L2-D4-T1-A	32.466	31.584	97.3	
S23-106642-L2-D5-T1-A	32.466	32.636	101	
S23-106642-L2-D6-T1-A	32.466	31.976	98.5	
S23-106642-L2-D3-T5-A	519.48	360.83	71.4[a]	85.8
S23-106642-L2-D3-T5-R	519.48	381.24		
S23-106642-L2-D4-T5-A	519.48	393.30	78.3[b]	
S23-106642-L2-D4-T5-R	519.48	420.49		
S23-106642-L2-D5-T5-A	519.48	509	98	
S23-106642-L2-D6-T5-A	519.48	496.66	95.6	

*Taken from study plan, [a] Mean of 69.5 and 73.4 %; [b] Mean of 75.7 and 80.9 %.

Analytical performance

Selectivity and Specificity

The analyte was determined in the final sample extracts by use of LC-MS/MS detection with evaluation of one (1) mass transition per analyte
A second mass transition was monitored for confirmation of peak identity but was not used for the quantification of target analyte.

Untreated samples for accompanying control sample work up, for determination of (concurrent) recoveries and, for preparation of matrix-matched calibration standards were supplied by the Test Site of the Analytical Phase.

The blank values at the expected retention time of the analyte resulting from reagents and/or the control sample material used for recovery determinations and for preparation of matrix-matched calibration standards did not exceed a level that would correspond to 30 % of the LOQ.
Correction for blank values was therefore not performed.
Example chromatograms representing control samples, the lowest calibration level, samples fortified at the LOQ and treated residue samples are included in **Błąd! Nie można odnaleźć źródła odwołania.**.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing peak areas of matrix-matched standards of 90 % matrix amount with solvent standards at comparable (or the same) nominal concentrations. Matrix effects were calculated as follows:

Matrix effect (%)	= [(100 · $A_{Matrix-Std}$) / ($A_{Solv-Std}$)] - 100
$A_{Solv-Std}$	Peak area of solvent standard
$A_{Matrix-Std}$	Peak area of matrix-matched standard

The matrix effects are summarised in the table below:

Matrix	**Standard Concentration (ng/mL)**	**Matrix Effect for Zoxamide (%)**	**Matrix Effect for Zoxamide (%)**
		Quantification (*m/z* 336 → 187)	**Mean Value**
Larval Diet containing 1.5 % acetone	0.300	-1.76	(-) 0.010
	0.500	0.334	
	1.00	1.33	
	2.00	-1.29	
	3.00	0.335	
	4.00	-0.853	
	5.00	0.351	
	6.00	1.48	

(+) matrix enhancement; (-) matrix suppression

The matrix suppression or enhancement was < 20 % for the investigated matrix and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the analytical phase.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.300 ng/mL to 6.00 ng/mL. This range corresponds to a mass fraction level of 0.619 mg/kg to 12.4 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. The calibration curve did not exceed two (2) orders of magnitude.

The calibration curves were linear and the regression residuals were randomly distributed. Furthermore, correlation coefficients (R) were ≥ 0.99. Linear regression was performed with 1/x-weighting.
Representative linear regression curves and residual plots are included in **Błąd! Nie można odnaleźć źródła odwołania.**.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. Standard solutions were distributed over the whole acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.
In order to still be within the linear calibration range, extracts of samples and recoveries at higher level were diluted by factors of 5and 100 with control sample extract.
Formula and an example calculation are part of the analytical method description given in **Błąd! Nie można odnaleźć źródła odwołania.**.

Method Validation

For the purpose of method validation, recoveries were conducted in accordance to SANTE/2020/12830, Rev. 2. prior sample analysis.
Five (5) recovery determinations at LOQ and five (5) recovery determinations at high level were performed for each analyte.
One (1) mass transition was evaluated and representative ion chromatograms along with the product ion mass spectrum are shown in **Błąd! Nie można odnaleźć źródła odwołania.** and D of the report. A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.
The following recoveries were obtained:

Matrix	Zoxamide Fortification level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Zoxamide (Mass Transition *m/z* m/z 336 → 187)					
Larval diet containing 1.5 % Acetone	2.97	93.4, 93.6, 93.8, 96.1, 94.8	94.4	1	5
	677	102, 97.7, 98.1, 98.1, 97.8	98.7	2	5

No observable peak was detected in any control sample extract
Recoveries are without any blank correction

Concurrent Recoveries

The analytical performance in terms of accuracy and repeatability was assessed for each analytical set by fortification of control (untreated) test portions of the respective matrix and subsequent determination of the concurrent recoveries upon applying the analytical method.
Each analytical set included at least the following types and numbers of samples:

- 1 reagent blank (sample work-up without matrix)
- 1 control samples (concurrent control)
- 2 control samples fortified at the LOQ (concurrent recoveries)
- 2 control samples fortified at higher level (concurrent recoveries)

Concurrent recoveries were handled and stored in the same way and for the same period of time as the sample extracts that were prepared within the same analytical set.
The following table summarises the obtained recoveries:

Zoxamide					
Matrix	**Zoxamide Fortification level (mg/kg)**	**Concurrent Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**
Zoxamide (Mass Transition *m/z* m/z 336 → 187)					
Larval diet containing 1.5 % Acetone	2.97	96.1, 96.7, 94.5, 94.7, 102	96.8	3	5
	677	101, 96.7, 96.7, 97.6, 102	98.9	3	5

No observable peak was detected in any control sample extract
Recoveries are without any blank correction

One (1) mass transition was evaluated and representative ion chromatograms along with the product ion mass spectrum are shown in **Błąd! Nie można odnaleźć źródła odwołania.** of the report.

Accuracy is reflected by the mean recovery per level while precision is reflected by the corresponding relative standard deviation.
All mean values at fortification levels of 2.97 mg zoxamide/kg (LOQ) and 677 mg zoxamide/kg for one (1) mass transition are within 70 % - 120 % with relative standard deviations ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2.

Stability of Stock and Fortification Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5).

Results were derived from GLP data available at the Test Site and are summarised below.

Analyte	**Solvent**	**Concentration (µg/mL)**	**Storage Period (Days)**	**Mean difference (in %) of stored stock solution compared to freshly prepared stock solution**
Zoxamide	Acetonitrile	1010	51	8

The mean peak area of the standard solution was within ± 10 % of the mean peak area of the freshly prepared solution indicating that stock solution is stable when stored at 1 °C to 10 °C in the dark for 51 days. This was sufficient to cover the length of time it was used in this study (10 days).

Stability of Final Extracts

The interval from preparation of the final extracts to injection did not exceed 24 hours. Due to the shortness of the interval any effect on the results due to a possible instability of the analyte in final sample extracts are considered to be insignificant.

Storage Stability

The maximum storage period from sampling to analysis was 23 days within this study. Residues are regarded as stable if the samples are stored deep-frozen for up to 30 days between sampling and analysis (OECD (2007), Test No 506: Stability of Pesticide Residues in Stored Commodities). Therefore, the proof of stability of the analytes in/on fortified or incurred samples upon storage in deep frozen conditions was not conducted as part of this analytical phase.

Conclusion

The method was successfully validated for determination of zoxamide in larval diet containing 1.5 % acetone with an LOQ of 2.97 mg zoxamide/kg and up to 677 mg zoxamide/kg according to guidance document(s) SANTE/2020/12830, rev. 2.
With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the samples of the study.

A 2.1.1.1.4 Non-target plants

A 2.1.1.1.4.1 Method validation

Comments of zRMS:	The method was successfully validated according to SANTE/2020/12830 rev. 2.

Reference:	KCP 10.6.2
Report	GLOB2013F: OECD Terrestrial Plant Test - Vegetative Vigour Test, Dewson, S., 2023, Stockbridge Technology Centre Ltd., Report No.: STC/22/E1557
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Method

The content of the active ingredient, zoxamide, in the spray solution was determined using a high performance liquid chromatography (HPLC) method based on conditions provided by the Sponsor. The analytical method was validated as part of Study STC/22/E1557 and also covers Study STC/22/E1558.

Calibration Standard Preparation

Zoxamide analytical standard (approximately 20 mg) was accurately weighed into glass weighing boats and quantitatively transferred to a 50 mL volumetric flask using acetonitrile (approximately 25 mL). Purified water (5 mL) was added prior to dilution to volume with further acetonitrile giving a primary stock solution (Cal A). A series of calibration standard solutions were then prepared by volumetric dilution with acetonitrile:water (9:1 v/v) as follows:

Solution diluted	Volume diluted (mL)	Final volume (mL)	Final solution
Cal A	8	10	Cal B
Cal A	6	10	Cal C
Cal A	8	20	Cal D
Cal A	2	10	Cal E

Cal A	1	10	Cal F
Cal A	1	20	Cal G
Cal A	1	50	Cal H

These were analysed to demonstrate linearity of the detector system prior to sample analysis.

An intermediate calibration solution (Cal D) was then used as a bracketing standard in the HPLC analysis of the verification and test sample solutions.

In order to assess the impact of any matrix effects a second Cal D solution was prepared as above but using diluent containing water supplied by the Test Facility.

Method validation

The accuracy and precision of the analytical procedure was verified by the analysis of laboratory prepared aqueous solutions containing known weights of the test item.

Five solutions were prepared at a level equivalent to approximately 110% of the highest treatment rate concentration. Test item (approximately 0.66 mL) was accurately weighed into separate 100 mL volumetric flasks. Purified water was used to disperse the test item and dilute the samples to volume.

Five solutions were also prepared at a level equivalent to approximately 20% of the highest treatment rate concentration. Test item (approximately 0.12 mL) was accurately weighed into separate 100 mL volumetric flasks. Purified water was used to disperse the test item and dilute the samples to volume.

Each flask was sonicated for 20 minutes with intermittent shaking and stirred for 5 minutes prior to sampling. The stirring was continued as aliquots were removed for analysis. Aliquots (10 mL) of each verification sample were then transferred to separate 100 mL volumetric flasks and diluted to volume with acetonitrile.

The resulting solutions were then filtered (0.45 µm, PTFE) into separate glass vials and the filtrates analysed by the HPLC method relative to a bracketing standard solution.

HPLC Conditions

The following conditions were used.

Instrument:	Agilent 1100 Liquid Chromatograph
Data handling system:	Chromeleon Thermo Version 7.2.4.8340
Column:	Zorbax Rx-C8, 5 µm (25 cm x 4.6 mm internal diameter)
Column temperature:	25°C
Mobile phase:	Acetonitrile:pH3 water[1] (8:2 v/v)
Flow rate:	0.5 mL/minute

Injection volume:	10 µL
Detector:	UV set at 254 nm
Retention time:	Approximately 10 minutes
Run time:	20 minutes

[1] Prepared by acidifying purified water with formic acid

Treatment of Data

From the data obtained by the HPLC analytical method, the content of the active ingredient was calculated by reference to the bracketing standard.

The individual response factor (RF) of the standards introduced immediately before and after the sample (bracketing standards) was calculated by the following equation:

$$RF = \frac{\text{standard concentration (mg/L)}}{\text{standard peak area}}$$

The average response factor (RF_{AVG}) of the bracketing standards was calculated and the concentration of the analyte in the analysed solution (C_A) is calculated from the following equation:

$$C_A \text{ (mg/L)} = RF_{AVG} \times \text{ sample peak area}$$

The concentration of the analyte in the test samples (C_B) was calculated from the following equation:

$$C_B \text{ (mg/L)} = C_A \text{ (mg/L)} \times \text{dilution factor}$$

where 10 is the dilution factor for the sample solution.

The recoveries were calculated from the equation:

$$\text{Recovery (\%)} = \frac{C_B \text{ (mg/L)}}{\text{intended concentration (mg/L)}} \times 100$$

For the method verification samples, the accuracy of the procedure was determined by calculating the recoveries from the assay of the five samples. The precision from the analysis of the verification samples was expressed as the relative standard deviation (RSD) of the recoveries determined for the samples.

Results and discussions

The data obtained from the analysis of the calibration standard solutions were used to prepare calibration curves by plotting the concentration of active ingredient versus the detector response (peak area) using least squares regression with no weighting. The detector was found to be linear over the range of standard solutions for zoxamide in acetonitrile:water (9:1 v/v) (Table 1, Figure 1) with a correlation coefficient of 1.0000.

The following recovery data was obtained from the analysis of the verification samples:

Matrix	Component	Verification level (mg/L)	n	Recovery (%)	RSD (%)
Water	Zoxamide	2824	5	103	0.7
		542.4	5	105	0.9

RSD - relative standard deviation

The raw data from the analysis are presented in Table 2. This data served to confirm that the method used was valid for the analysis of the spray solution samples.

No significant matrix effect was observed between the calibration solutions prepared using laboratory water and water supplied by the Test Facility as the calculated matrix effect was ≤ 20%. The matrix effect was calculated as follows:

$$\text{Matrix effect (\%)} = 100 \times \frac{\text{peak area (matrix)}}{\text{peak area}} - 100$$

The raw data from the analysis are presented in Table 3.

The specificity of the analytical method was confirmed by retention time match and by diode array analysis. The UV spectra of the analytes in a standard solution and a spray solution sample are presented in Figure 2. In addition analysis of the dilution solvent confirmed that it did not contain components that would interfere with the analysis.

Table 1 Standard calibration for zoxamide

Standard concentration (mg/L)	Peak area	Accuracy (%)
400.2	135.43	100
320.2	108.03	100
240.1	81.273	100
160.1	54.594	101
80.04	27.042	99
40.02	14.119	103
20.01	6.9931	100
8.004	2.8190	96

Linear regression (including x = 0, y = 0) $y = 0.3376x + 0.225$, $r = 1.0000$

x = concentration
y = peak area

Figure 1 Standard calibration for zoxamide

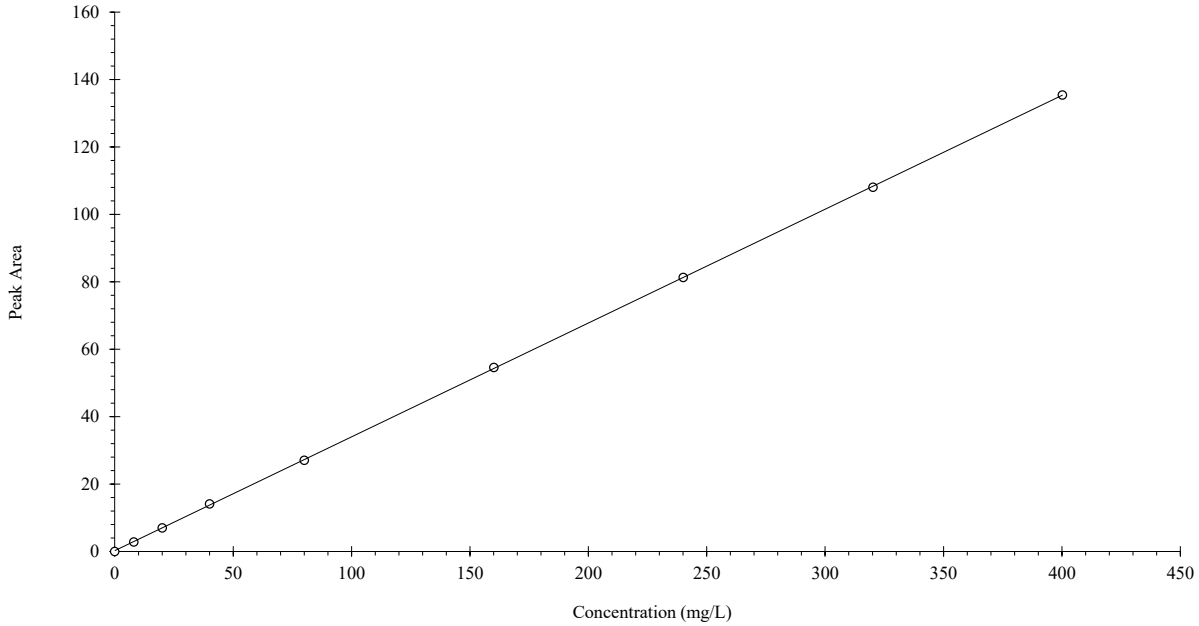


Table 2 Analysis of method verification samples for the determination of zoxamide

Sample	Peak area	C_A (mg/L)	Dilution factor	C_B (mg/L)	Concentration of fortified solution (mg/L)	Recovery (%)
160.1mg/L std	54.711	-	-	-	-	-
High spike A	99.635	291.8	10	2918	2812	103.8
High spike B	99.734	292.1	10	2921	2859	102.2
High spike C	97.720	286.2	10	2862	2803	102.1
High spike D	99.075	290.1	10	2901	2811	103.2
160.1 mg/L std	54.625	-	-	-	-	-
High spike E	99.631	292.2	10	2922	2837	103.0
LOQ spike A	19.370	56.80	10	568.0	538.5	105.5
LOQ spike B	19.843	58.19	10	581.9	557.4	104.4
LOQ spike C	19.270	56.51	10	565.1	532.7	106.1
160.1 mg/L std	54.565	-	-	-	-	-
LOQ spike D	19.531	57.20	10	572.0	550.3	103.9
LOD spike E	19.262	56.42	10	564.2	533.2	105.8
160.1 mg/L std	54.761	-	-	-	-	-

Mean recovery - High fortifications = 103% (RSD = 0.7%)
Mean recovery - LOQ fortifications = 105% (RSD = 0.9%)

Table 3 Analysis of calibration solutions for the determination of matrix effects

Analyte	Sample	Peak area	Mean Peak Area	Matrix effect
Zoxamide	160.1 mg/L std	54.165	54.325	-0.02
	160.1 mg/L std	54.484		
	Matrix – 160.1 mg/L std	54.324	54.313	
	Matrix – 160.1 mg/L std	54.302		

Conclusion

The method was demonstrated to be suitable for the analysis of aqueous solutions containing GLOB2013F.

The content of the active ingredient in the provided spray solutions was found to be within the acceptable range (70-120% of the theoretical content).

A 2.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

A 2.1.2.1.1 Validation of Analytical Methods to Determine Residues of Zoxamide in Plant Matrices

A 2.1.2.1.1.1 Method validation

Comments of zRMS:	The method was fully validated according to the requirements of SANTE/2020/12830 Rev.1. It can be used to determine the residues of zoxamide in different crop matrices (potato (tuber), grape (bunches, juice, raisin, wine), lettuce, dry bean (seeds) and oilseed rape (seeds)) with LOQ 0.01 mg/kg.

Reference:	KCA 4.1.2
Report	Validation of Analytical Methods to Determine Residues of Zoxamide in Plant Matrices, Gustloff, C., 2022, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07039
Guideline(s):	SANTE/2020/12830 Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, for zoxamide samples of potato (tuber), grape (bunches, juice, raisin, wine), lettuce, dry bean (seeds) and oilseed rape (seeds) were extracted with acetonitrile and if necessary, after addition of water. For oilseed rape (seeds) purification of an aliquot of the acetonitrile extract was performed by freezing out at ≤ 18 °C followed by dispersive SPE with PSA and magnesium sulfate. Quantification was performed by use of LC MS/MS detection.

Results and discussions

Selectivity

Two (2) mass transitions were evaluated thus demonstrating a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or the control sample extracts of each matrix, thus demonstrating a high level of selectivity.

Matrix Effects

Matrix effects on the detection of zoxamide in extracts of potato (tuber) were found to be significant (≥ 20 %). Therefore, matrix-matched standards were used for quantification. Matrix effects on the detection of zoxamide in extracts of grape (bunches, juice, raisin, wine), lettuce, dry bean (seeds) and oilseed rape (seeds) were found to be insignificant (< 20 %). However, matrix-matched standards were used for quantification.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of seven (7) concentration levels ranging from 0.3 ng/mL to 30 ng/mL. This range corresponds to 0.003 mg/kg to 0.3 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract.
Linear regression was performed with 1/x weighting. The calibration curves obtained for both mass transitions were linear since the regression residuals were randomly distributed. Furthermore, coefficients of determination (R^2) were ≥ 0.98.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was spread over the whole analytical sequence. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ is the lowest validated fortification level for zoxamide and was successfully established at 0.01 mg/kg in potato (tuber), grape (bunches, juice, raisin, wine), lettuce, dry bean (seeds) and oilseed rape (seeds) for the two (2) mass transitions.
The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for all matrices, which is 30 % of the LOQ.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

The following recoveries were obtained:

Zoxamide							
Mass Transition	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Potato (tuber)							
m/z 336→187*	0.01	99, 92, 88, 90, 88	91	4.8	5	92	3.3
	0.1	94, 93, 93, 92, 91	92	1.0	5		
m/z 336→159	0.01	94, 90, 85, 88, 84	88	4.6	5	89	4.0
	0.1	92, 89, 92, 85, 93	90	3.6	5		
Grape (bunches)**							

m/z 336→187*	0.01	100, 95, 100, 92, 93	96	3.7	5	100	5.5
	0.1	104, 109, 101, 106, 103	105	3.1	5		
m/z 336→159	0.01	100, 94, 99, 95, 99	97	2.6	5	101	4.9
	0.1	106, 108, 104, 106, 105	106	1.4	5		
Grape (juice)							
m/z 336→187*	0.01	99, 103, 101, 98, 103	101	2.3	5	103	3.6
	0.1	107, 104, 104, 99, 110	105	3.8	5		
m/z 336→159	0.01	99, 100, 94, 98, 102	98	3.0	5	101	4.5
	0.1	104, 110, 100, 100, 103	104	4.2	5		
Grape (raisin)							
m/z 336→187*	0.01	116, 112, 109, 115, 110	113	2.8	5	114	3.0
	0.1	120, 115, 116, 113, 111	115	3.0	5		
m/z 336→159	0.01	116, 112, 108, 110, 107	111	3.4	5	112	3.2
	0.1	118, 114, 114, 114, 110	114	2.4	5		
Grape (wine)							
m/z 336→187*	0.01	84, 81, 89, 79, 80	83	4.6	5	86	5.1
	0.1	88, 87, 89, 90, 91	89	1.9	5		
m/z 336→159	0.01	81, 83, 81, 81, 80	81	1.4	5	84	4.5
	0.1	88, 85, 87, 87, 91	88	2.4	5		
Lettuce							
m/z 336→187*	0.01	109, 107, 96, 96, 97	101	6.4	5	105	6.2
	0.1	114, 113, 105, 104, 105	108	4.2	5		
m/z 336→159	0.01	112, 105, 98, 92, 99	101	7.7	5	104	6.8
	0.1	114, 112, 107, 103, 101	107	5.0	5		
Dry bean (seeds)							
m/z 336→187*	0.01	118, 112, 116, 119, 106	114	4.7	5	117	4.0
	0.1	120, 118, 118, 121, 119	119	1.2	5		
m/z 336→159	0.01	118, 115, 116, 118, 106	115	4.3	5	116	3.3
	0.1	120, 114, 114, 117, 118	117	2.1	5		
Oilseed rape (seeds)							
m/z 336→187*	0.01	81, 80, 75, 73, 62	74	10	5	77	7.7
	0.1	81, 80, 79, 77, 79	79	2.0	5		
m/z 336→159	0.01	76, 82, 71, 70, 76	75	6.7	5	76	5.1
	0.1	79, 77, 77, 74, 81	78	3.1	5		

Recoveries are without any blank correction

* Proposed to be used for quantification

** Blank correction was performed (residue of corresponding control sample below LOD); uncorrected recoveries can be found in Appendix C

All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1 (see table below).

Concentration Level (mg/kg)	**Range of Mean Recoveries (%)**	**Precision, Rel. Std. Dev. (%)**
≤ 0.01	60 - 120	30
> 0.01 - ≤ 0.1	70 - 120	20

Stability of Analyte in Working Solutions

Zoxamide was found to be stable for 164 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark.
Zoxamide was found to be stable for 13 days when prepared in acetonitrile/0.1 % formic acid (1+1, v+v) and stored at typically1 °C to 10 °C in the dark.

Stability of Analyte in Sample Extracts

Zoxamide was found to be stable in final extracts of potato (tuber), grape (bunches, juice, wine) and lettuce for 11 days when stored at typically 1 °C to 10 °C in the dark. Zoxamide was found to be stable in final extracts of grape (raisin) and dry bean (seeds) for 12 days when stored at typically 1 °C to 10 °C in the dark and in final extracts of oilseed rape (seeds) for 7 days.

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring, for the determination of zoxamide in potato (tuber), grape (bunches, juice, raisin, wine), lettuce, dry bean (seeds) and oilseed rape (seeds) with the tested LOQ of 0.01 mg/kg.

A 2.1.2.1.1.2 Method validation RH-141452 and RH-141455

Comments of zRMS:	The method was fully validated according to the requirements of SANTE/2020/12830 Rev.1. It can be used to determine the residues of zoxamide metabolites: RH-1452 in grape (bunches, juice, raisin and wine) and potato matrices with LOQ 0.01 mg/kg and RH-1455 in potato (tuber) with LOQ 0.01 mg/kg.

Reference:	KCA 4.1.2
Report	Validation of an Analytical Method to Determine Residues of Zoxamide Metabolites (RH-1452 and RH-1455) in Grape and Potato Matrices, Gustloff, C., 2022, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07040
Guideline(s):	SANTE/2020/12830 Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, for RH-1452 and RH-1455 samples of potato (tuber) and grape (bunches, juice, raisin and wine) were extracted with glycine buffer. Liquid-liquid partition was performed twice with acetonitrile. Clean-up of the extract was performed with an ENVI-Carb SPE cartridge. Quantification was performed by use of LC-MS/MS.

Results and discussions

Selectivity

Two (2) mass transitions were evaluated thus demonstrating a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or the control sample extracts of each matrix, thus demonstrating a high level of selectivity.

Matrix Effects

Matrix effects were ≥ ± 20 % and deemed to be significant for RH-1452 in potato (tuber).
Matrix suppression or enhancement was < 20 % for RH-1452 in grape (bunches, juice, raisin and wine) and for RH-1455 in potato (tuber) and thus deemed to be insignificant.
However, matrix-matched standards were used for quantification throughout the study.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.60 ng/mL to at least 50 ng/mL. This range corresponds to 0.003 mg/kg to 0.25 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract.
Linear regression was performed with 1/x weighting. The calibration curves obtained for both mass transitions and all matrices were linear since the regression residuals were randomly distributed. Furthermore, coefficients of determination (R^2) were ≥ 0.98.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was spread over the whole analytical sequence. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ is the lowest validated fortification level for each analyte and was successfully established at 0.01 mg/kg in potato (tuber) and grape (bunches, juice, raisin and wine) for the two (2) mass transitions.
The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for all matrices, which is 30 % of the LOQ.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item(s) and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). The analytes were fortified jointly and quantified separately. The following recoveries were obtained:

RH-1452							
Mass Transition	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Potato (tuber)							
m/z 219→175*	0.01	95, 96, 93, 98, 96	95	1.5	5	90	8.0
	0.1	83, 77, 81, 85, 93	84	6.9	5		
m/z 221→147	0.01	94, 95, 96, 102, 96	97	3.4	5	91	8.7
	0.1	82, 77, 84, 86, 94	85	7.2	5		

Grape (bunches)							
m/z 219→175*	0.01	95, 97, 88, 102, 101	96	5.6	5	92	9.1
	0.1	100, 91, 76, 86, 85	88	10	5		
m/z 221→147	0.01	91, 94, 84, 99, 100	94	7.0	5	90	9.0
	0.1	98, 88, 75, 84, 85	86	9.5	5		
Grape (juice)							
m/z 219→175*	0.01	100, 89, 93, 96, 107	97	7.3	5	92	9.5
	0.1	92, 75, 85, 93, 86	86	8.1	5		
m/z 221→147	0.01	102, 93, 94, 95, 100	97	4.2	5	92	7.8
	0.1	89, 78, 85, 94, 87	87	6.6	5		
Grape (raisin)							
m/z 219→175*	0.01	92, 96, 95, 102, 88	94	5.5	5	91	6.7
	0.1	85, 90, 97, 88, 81	88	6.7	5		
m/z 221→147	0.01	92, 92, 84, 100, 88	91	6.6	5	89	6.6
	0.1	86, 91, 88, 86, 78	86	5.6	5		
Grape (wine)							
m/z 219→175*	0.01	74, 61, 92, 90, 72	78	17	5	82	15
	0.1	77, 95, 90, 75, 97	87	12	5		
m/z 221→147	0.01	70, 61, 95, 89, 71	77	19	5	82	16
	0.1	75, 95, 90, 81, 98	88	11	5		

No observable peak was detected in any control sample extract

Recoveries are without any blank correction

* Proposed to be used for quantification

RH-1455							
Mass Transition	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Potato (tuber)							
m/z 233→109*	0.01	99, 99, 98, 106, 84	97	8.2	5	91	10
	0.1	84, 77, 82, 85, 96	85	8.3	5		
m/z 233→153	0.01	95, 93, 91, 105, 83	93	8.5	5	89	10
	0.1	83, 75, 82, 84, 96	84	9.3	5		

No observable peak was detected in any control sample extract

Recoveries are without any blank correction

* Proposed to be used for quantification

All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1 (see table below).

Concentration Level (mg/kg)	**Range of Mean Recoveries (%)**	**Precision, Rel. Std. Dev. (%)**
≤ 0.01	60 - 120	≤ 30
> 0.01 - ≤ 0.1	70 - 120	≤ 20

Stability of Analytes in Working Solutions

RH-1452 was found to be stable for 968 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark.
RH-1452 was found to be stable for 11 days when prepared in acetonitrile/water (2+8, v+v) and stored at typically 1 °C to 10 °C in the dark.
RH-1455 was found to be stable for 2277 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark.
RH-1455 was found to be stable for 11 days when prepared in acetonitrile/water (2+8, v+v) and stored at typically 1 °C to 10 °C in the dark.

Stability of Analytes in Sample Extracts

RH-1452 was found to be stable in final extracts of all matrices when stored at typically 1 °C to 10 °C in the dark for at least 10 days for Potato (tuber), for at least 11 days in Grape (bunches), for at least 9 days in Grape (raisin and wine) and for at least 7 days in Grape (juice).
RH-1455 was found to be stable in final extracts of Potato (tuber) when stored at typically 1 °C to 10 °C in the dark for at least 10 days.

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring for the determination of RH-1452 and RH-1455 in potato (tuber) and grape (bunches, juice, raisin and wine) with the tested LOQ of 0.01 mg/kg.

A 2.1.2.1.1.3 Method validation RH-150721

Comments of zRMS:	The method was fully validated according to the requirements of SANTE/2020/12830 Rev.1. It can be used to determine the residues of zoxamide metabolite RH-150721 in wine with LOQ 0.01 mg/kg.

Reference:	KCA 4.1.2
Report	Validation of an Analytical Method for Determination of Zoxamide Metabolite RH-150721 in Wine, Gustloff, C., 2022, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07042
Guideline(s):	SANTE/2020/12830 Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, samples of grape (wine) were extracted with 1 % potassium hydrogen carbonate. Clean-up of the extract was performed by SPE with an OASIS HLB cartridge. Quantification was performed by use of LC-MS/MS.

Results and discussions

Selectivity

Two (2) mass transitions were evaluated thus demonstrating a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or the control sample extracts of grape (wine), thus demonstrating a high level of selectivity.

Matrix Effects

Matrix effects on the detection of RH-150721 in extracts of grape (wine) were found to be insignificant (< 20 %). However, matrix-matched standards were used for quantification.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of eight (8) concentration levels ranging from 1.5 ng/mL to 150 ng/mL. This range corresponds to 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract.
Linear regression was performed with 1/x weighting. The calibration curves obtained for both mass transitions were linear since the regression residuals were randomly distributed. Furthermore, coefficients of determination (R^2) were ≥ 0.98.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was spread over the whole analytical sequence. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ is the lowest validated fortification level for RH-150721 and was successfully established at 0.01 mg/kg in grape (wine) for the two (2) mass transitions.
The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for grape (wine), which is 30 % of the LOQ.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). The following recoveries were obtained:

RH-150721							
Mass Transition	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Grape (wine)							
m/z 318→187*	0.01	77, 75, 73, 71, 73	74	3.4	5	77	5.9
	0.1	78, 73, 80, 85, 83	80	5.6	5		
m/z 318→159	0.01	79, 76, 73, 75, 75	76	2.9	5	78	5.9
	0.1	76, 73, 79, 87, 83	80	7.2	5		

No observable peak was detected in any control sample extract

Recoveries are without any blank correction

* Proposed to be used for quantification

All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1 (see table below).

Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)
≤ 0.01	60 - 120	≤ 30
> 0.01 - ≤ 0.1	70 - 120	≤ 20

Stability of Analyte in Working Solutions

RH-150721 was found to be stable for at least 7 days when prepared in 1 % acetic acid in methanol / water (4+6, v+v) and stored at typically 1 °C to 10 °C in the dark.
RH-150721 was found to be stable for at least 40 days when prepared in methanol and stored at typically1 °C to 10 °C in the dark.

Stability of Analyte in Sample Extracts

RH-150721 was found to be stable in final extracts of grape (wine) for at least 7 days when stored at typically 1 °C to 10 °C in the dark.

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring for the determination of RH-150721 in grape (wine) with the tested LOQ of 0.01 mg/kg.

A 2.1.2.1.2 Extraction efficiency

Comments of zRMS:	The study is accepted.

Reference:	KCA 4.1.2
Report	Determination of Extraction Efficiency by Comparison of Methods for [14C]Zoxamide in Grape Plants, Maric, A., 2023, Eurofins Agroscience Services Ecochem Gmbh, Report No.: S23-100483
Guideline(s):	SANTE/2017/10632, rev. 3, 22 November 2017
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Homogenised grape leaves and grape fruits treated with the substance [phenyl-U-^{14}C]Zoxamide originating from a grape plants metabolism study of Eurofins Agroscience Services EcoChem GmbH (Study S22-01899) were investigated. The following table shows details of the test material.

EAS Sample ID	Originating from Study	Description
221899A11	S22-01899	Homogenised grape leaves
221899A21		Homogenised grape fruits

Grape leaves and grape fruits were extracted with methods listed below:

Commodity	Method
Grape leaves	Residue Extraction Method 1 (QuEChERS, based on S21-07039)
Grape fruits	Residue Extraction Method 1 (QuEChERS, based on S21-07039)
Grape fruits	Residue Extraction Method 2 (based on S21-07040)
Grape leaves	Residue Extraction Method 3 (modified QuEChERS method)

Extraction with **Residue Method 1** was performed by extraction of the homogenised grape leaves and fruits with acetonitrile (sample/solvent ratio 1/1, w/v). Afterwards the acetonitrile phase was separated from the aqueous phase using a mixture of salts. The acetonitrile extract was analysed further.
Extraction with **Residue Method 2** was performed by extraction of the homogenised grape fruits with 1 M glycine buffer (sample/solvent ratio 1/10, w/v) followed by addition of water (sample/solvent ratio 1/9, w/v) and by addition of 3 M hydrochloric acid (sample/solvent ratio 1/5 w/v).
Extraction with **Residue Method 3** was performed by extraction of the homogenised grape leaves with acetonitrile (sample/solvent ratio 1/1, w/v). Afterwards the acetonitrile phase was separated from the aqueous phase using a mixture of salts. The extraction was repeated twice without addition of salts. The acetonitrile extracts were combined and analysed further.

Results and discussions/Conclusion

Extraction efficiency (extracted radioactivity) for grape leaves based on the TRR value in mg eq/kg of the extracted radioactivity of Residue Method 1 and 3 compared to Metabolism Method from Study S22-01899 amounted to 44.2% and 87.9%, respectively. Extraction rates for both methods are given in the table below.

Grape leaves	Metabolism Method*		Residue Method 1			Residue Method 3		
	% TRR	mg eq/kg	% TRR	mg eq/kg	%**	% TRR	mg eq/kg	%**
	100.0	35.388	100.0	32.792		100.0	36.140	
Extraction	99.2	35.107	47.3	15.526	**44.2**	85.4	30.847	**87.9**

*data transcribed from Study "Metabolism of [^{14}C]Zoxamide in Grape Plants" at Eurofins Agroscience Services EcoChem GmbH (Study S22-01899)
** Extraction rates based on TRR value in mg eq/kg determined with Residue Method 1 or 3 compared to Metabolism Method from Study S22-01899

Zoxamide was detected in all extracts of grape leaves. The amount of extracted Zoxamide accounted for 44.4% of TRR (14.572 mg eq/kg) for Residue Method 1 and 81.6% of TRR (29.501 mg eq/kg) for Residue Method 3. This corresponds to extraction efficiency of Zoxamide of 42.6% for Residue Method 1 and

86.3% for Residue Method 3. An overview of the extraction efficiency of Zoxamide in grape leaves is given in the table below.

Grape leaves	Metabolism Method*		Residue Method 1			Residue Method 3		
	% TRR	mg eq/kg	% TRR	mg eq/kg	%**	% TRR	mg eq/kg	%**
	100.0	35.388	100.0	32.792		100.0	36.140	
Analysed extracts	99.2	35.104	47.0	15.428		85.1	30.762	
Zoxamide	**96.6**	**34.181**	**44.4**	**14.572**	**42.6**	**81.6**	**29.501**	**86.3**

*data transcribed from Study "Metabolism of [^{14}C]Zoxamide in Grape Plants" at Eurofins Agroscience Services EcoChem GmbH (Study S22-01899).
**extraction efficiency based on TRR value in mg eq/kg determined with Residue Method 1 or 3 compared to Metabolism Method from Study S22-01899

Low extraction efficiency of Residue Method 1 could be caused by oversaturation of the extract as residue levels in the grape leaves sample are high. Residue Method 3 was developed during this study. This method is based on Residue Method 1 but uses a higher amount of solvent. Sufficient extraction efficiency was achieved with Residue Method 3. Therefore, it can be concluded, that the amount of solvent used for extraction with Residue Method 1 was not sufficient to extract the residues, since both methods differ only regarding the amount of solvent.
Extraction rates (extracted radioactivity) for grape fruits based on the TRR value in mg eq/kg of the extracted radioactivity of Residue Method 1 and 2 compared to Metabolism Method from Study S22-01899 amounted to 76.8% and 23.9%, respectively. Extraction rates for both methods are given in the table below.

Grape fruits	Metabolism Method*		Residue Method 1			Residue Method 2		
	% TRR	mg eq/kg	% TRR	mg eq/kg	%**	% TRR	mg eq/kg	%**
	100.0	1.044	100.0	0.911		100.0	0.849	
Extraction	99.5	1.038	87.5	0.797	**76.8**	29.3	0.248	**23.9**

*data transcribed from Study "Metabolism of [^{14}C]Zoxamide in Grape Plants" at Eurofins Agroscience Services EcoChem GmbH (Study S22-01899)
** Extraction rates based on TRR value in mg eq/kg determined with Residue Method 1 or 2 compared to Metabolism Method from Study S22-01899
Zoxamide was detected in all extracts of grape fruits. The amount of extracted Zoxamide accounted for 82.9% of TRR (0.755 mg eq/kg) for Residue Method 1 and 24.3% of TRR (0.206 mg eq/kg) for Residue Method 2. This corresponds to extraction efficiency of Zoxamide of 75.6% for Residue Method 1 and 20.7% for Residue Method 2.
An overview of the extraction efficiency of Zoxamide in grape fruits is given in the table below.

Grape fruits	Metabolism Method*		Residue Method 1			Residue Method 2		
	% TRR	mg eq/kg	% TRR	mg eq/kg	%**	% TRR	mg eq/kg	%**
	100.0	1.044	100.0	0.911		100.0	0.849	
Analysed extracts	99.0	1.033	85.6	0.780		29.3	0.248	
Zoxamide	**95.6**	**0.998**	**82.9**	**0.755**	**75.6**	**24.3**	**0.206**	**20.7**
RH-141452	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.a.**	**n.d.**	**n.d.**	**n.a.**

n.d.: not detected
n.a.: not applicable
*data transcribed from Study 'Metabolism of [^{14}C]Zoxamide in Grape Plants" at Eurofins Agroscience Services EcoChem GmbH (Study S22-01899).
**extraction efficiency based on TRR value in mg eq/kg determined with Residue Method 1 or 2 compared to Metabolism Method from Study S22-01899

Residue Method 2 was intended to extract the metabolite RH-141452. This metabolite was not detected in grape fruits using any of the Residue Methods. It was also not found using Metabolism Method in study S22-01899. The method was not intended to extract the parent compound Zoxamide. The low extraction efficiency indicates that this method is not suitable for Zoxamide extraction, but no conclusion can be made on the suitability of this method for the extraction of the metabolite RH-141452.

A 2.1.2.1.3 Honey

A 2.1.2.1.3.1 Method validation

Comments of zRMS: The method was fully validated according to the requirements of SANTE/2020/12830 Rev.2. It can be used to determine the residues of zoxamide and its metabolites RH-1452, RH-1455 and RH-150721 in honey with LOQ 0.01 mg/kg.

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The following recoveries were obtained:

Honey							
Mass Transition	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Propamocarb-HCL							
m/z 189→74*	0.01	97, 95, 100, 89, 86	93	6.0	5	86	12
	0.1	72, 75, 70, 86, 87	78	10	5		
m/z 189→144	0.01	94, 94, 97, 91, 85	92	5.1	5	86	11
	0.1	73, 74, 71, 86, 91	79	11	5		
Zoxamide							
m/z 336→187*	0.01	104, 108, 102, 108, 101	104	3.0	5	100	5.4
	0.1	93, 97, 97, 99, 93	96	2.7	5		
m/z 336→159	0.01	106, 108, 104, 101, 97	103	4.1	5	100	5.1
	0.1	93, 96, 93, 100, 101	96	3.8	5		
RH-1452							
m/z 219→175*	0.01	101, 102, 87, 97, 90	95	7.1	5	97	6.1
	0.1	100, 92, 100, 105, 100	99	4.8	5		
m/z 221→147	0.01	97, 99, 80, 96, 94	93	8.2	5	95	6.4
	0.1	96, 90, 97, 100, 99	96	4.3	5		
RH-1455							
m/z 233→109*	0.01	92, 97, 77, 79, 80	85	11	5	89	8.7
	0.1	93, 88, 92, 95, 95	93	3.4	5		
m/z 233→153	0.01	86, 95, 79, 86, 88	87	6.6	5	89	5.7
	0.1	91, 87, 90, 96, 93	91	3.7	5		
RH-150721							
m/z 318→187*	0.01	90, 83, 71, 80, 62	77	14	5	79	11
	0.1	87, 84, 82, 78, 72	81	6.8	5		
m/z 318→159	0.01	88, 84, 68, 74, 60	75	15	5	78	12
	0.1	88, 83, 82, 80, 73	81	6.5	5		

Reference: KCA 4.1.2

Report: Validation of Analytical Methods for Determination of Propamocarb-HCl, Zoxamide and its metabolites RH-1452, RH-1455 and RH-150721 in Honey, Gustloff, C., 2023, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100692

Guideline(s):	SANTE/2020/12830 Rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, for zoxamide and propamocarb-HCl samples were extracted with acetonitrile and if necessary, after addition of water. The ratio was 10 mL of extraction solvent per 1.0 g of honey. Quantification was performed by use of LC-MS/MS.
In brief, for RH-1452 and RH-1455 samples were extracted with glycine buffer. Liquid-liquid partition was performed twice with ethylacetate. Clean-up of the extract was performed with an ENVI-Carb SPE cartridge. Quantification was performed by use of LC-MS/MS.
In brief, for RH-150721 samples were extracted twice with methanol. Both extracts were combined, evaporated and resolved in final solvent. Quantification was performed by use of LC-MS/MS

Results and discussions/Conclusion

Item	Activity, Result, Assessment
Study Scope	Validation of an analytical method according to guidance documents SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analytes	Propamocarb-HCl, Zoxamide and its metabolite RH-1452, RH-1455 and RH-150721
Matrix	Honey
Method Reference	Zoxamide and Propamocarb-HCl: Based on Multi-residue method QuEChERS [1] as validated for crops in S21-07039 [2] for Zoxamide RH-1452 and RH-1455: Based on Podhorniak, L.V. (2014) [3] as validated for potato and grape in S21-07040 [4] RH-150721: Developed and validated in the current study
LOQ	0.01 mg/kg (lowest validated fortification level)
LOD	30 % of the LOQ (lowest calibration standard)
Principle of the Analytical Procedure	Homogenisation: Stirring with spatula Propamocarb-HCl + Zoxamide: Extraction: Acetonitrile and addition of water, shaking Clean-up: none Reconstitution: Acetonitrile/0.1 % formic acid in water (1+1, v+v) Sample concentration in final extract: 0.1 g sample per mL of extract RH-1452 + RH-1455: Extraction: glycin buffer, shaking Liquid/liquid partition: ethyl acetate Clean-up: GCB SPE cartridge Reconstitution: Acetonitrile/ Water (2+8, v+v) Sample concentration in final extract: 0.2 g sample per mL of extract

<table>
<tr><th>Item</th><th>Activity, Result, Assessment</th></tr>
<tr><td></td><td>RH-150721:
Extraction: Methanol, shaking
Clean-up: none
Reconstitution: 1 % acetic acid in methanol / water (4+6, v+v)
Sample concentration in final extract: 0.2 g sample per mL of extract
Quantification: LC-MS/MS</td></tr>
<tr><td>Selectivity and Specificity</td><td>Demonstrated by validation of two (2) mass transitions
Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.</td></tr>
<tr><td>Matrix Effects on Analyte Detection</td><td>Significant (≥ 20 %) for RH-150721
Insignificant (< 20 %) for Propamocarb-HCl, Zoxamide and its metabolites RH-1452 and RH-1455</td></tr>
<tr><td>Calibration</td><td>Matrix-matched calibration standards
A minimum of five (5) concentration levels
Single determination
Injection of standard solutions spread over the whole acquisition batch
Propamocarb-HCl + Zoxamide:
Concentration range: 0.3 ng/mL to 30 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg
RH-1452 + RH-1455:
Concentration range: 0.6 ng/mL to 60 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg
RH-150721:
Concentration range: 0.6 ng/mL to 60 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg

Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract
The validated range does not exceed two (2) orders of magnitude</td></tr>
<tr><td>Quantification</td><td>Linear regression with 1/x weighting
Regression residuals randomly distributed
Coefficients of determination (R^2) ≥ 0.99</td></tr>
<tr><td>Accuracy and Precision</td><td>Five (5) fortifications at 0.01 mg/kg (LOQ)
Five (5) fortifications at 0.1 mg/kg (10x LOQ)
RH-1452 and RH-1455 were fortified jointly and quantified separately. Zoxamide and Propamocarb-HCl were fortified jointly and quantified separately. RH-150721 was fortified and quantified separately.
Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2:
<table>
<tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr>
</table>
</td></tr>
</table>

Item	Activity, Result, Assessment
	≤ 0.01 | 60 - 120 | ≤ 30 > 0.01 - ≤ 0.1 | 70 - 120 | ≤ 20
Stability of Analytes in Standard Solutions	Zoxamide: Within ± 10 % for 186 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 7 days when prepared in 0.1 % formic acid in water/acetonitrile (1+1, v+v) and stored at typically1 °C to 10 °C in the dark. Propamocarb-HCl: Within ± 10 % for 30 days when prepared in 1M HCl and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 7 days when prepared in 0.1 % formic acid in water/acetonitrile (1+1, v+v) and stored at typically1 °C to 10 °C in the dark. RH-1452 + RH-1455: Within ± 10 % for 186 days for RH-1452 and 214 days for RH-1455 when prepared in methanol and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 22 days when prepared in water/acetonitrile (8+2, v+v) and stored at typically1 °C to 10 °C in the dark. RH-150721: Within ± 10 % for 172 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 7 days when prepared in 0.1 % acetic acid in methanol/ water (4+6, v+v) and stored at typically1 °C to 10 °C in the dark.
Stability of Analytes in Sample Extracts	Recoveries within 70 % - 120 % in honey for at least 7 days when stored at typically 1 °C to 10 °C in the dark.
Conclusion	The method was found to be valid according to the guidance documents SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring

A 2.1.2.1.3.2 Independent laboratory validation

Comments of zRMS:	The method was fully validated according to the requirements of SANTE/2020/12830 Rev.2. It can be used to determine the residues of zoxamide and its metabolites RH-1452, RH-1455 and RH-150721 in honey with LOQ 0.01 mg/kg.

The following recoveries were obtained:

Zoxamide

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition *m/z* 336→187 (Proposed for Quantification)							
Honey (multi-flower)	0.01	93, 99, 93, 93, 96	95	3.0	5	94	6.6
	0.1	100, 103, 80, 97, 92	94	9.4	5		
Mass Transition *m/z* 336→159 (Proposed for Confirmation)							
Honey (multi-flower)	0.01	91, 97, 97, 91, 96	94	3.1	5	94	6.6
	0.1	99, 103, 80, 97, 90	94	9.5	5		

RH-1452

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition *m/z* 219→175 (Proposed for Quantification)							
Honey (multi-flower)	0.01	82, 80, 78, 89, 88	83	5.7	5	82	4.1
	0.1	84, 82, 82, 80, 81	82	1.6	5		
Mass Transition *m/z* 221→147 (Proposed for Confirmation)							
Honey (multi-flower)	0.01	87, 85, 75, 87, 88	84	6.3	5	83	4.5
	0.1	82, 82, 84, 82, 82	82	1.3	5		

RH-1455

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition *m/z* 233→109 (Proposed for Quantification)							
Honey (multi-flower)	0.01	70, 70, 77, 81, 83	76	8.1	5	76	5.6
	0.1	78, 76, 78, 78, 75	77	2.0	5		
Mass Transition *m/z* 233→153 (Proposed for Confirmation)							
Honey (multi-flower)	0.01	72, 72, 74, 82, 80	76	6.1	5	76	4.6
	0.1	78, 77, 81, 76, 76	77	3.0	5		

RH-150721

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition *m/z* 318→187 (Proposed for Quantification)							
Honey (multi-flower)	0.01	84, 81, 80, 83, 82	82	1.7	5	87	6.5
	0.1	92, 96, 92, 92, 87	92	3.5	5		
Mass Transition *m/z* 318→159 (Proposed for Confirmation)							
Honey (multi-flower)	0.01	86, 82, 81, 85, 84	83	2.3	5	87	5.7
	0.1	92, 95, 92, 93, 86	91	3.6	5		

Reference: KCA 4.1.2

Report: Independent Laboratory Validation of Analytical Methods for Determination of Propamocarb-HCl, Zoxamide and its Metabolites in Honey, Asekunowo, J., 2024, Eurofins Agroscience Services EAG Laboratories GmbH, Report No.: S23-100694

Guideline(s): SANTE/2020/12830, rev. 2

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Summary

Item	Activity, Result, Assessment
Study Scope	Independent Laboratory Validation of an analytical method according to guidance document SANTE/2020/12830, rev. 2 for monitoring
Analytes	Propamocarb-HCl, Zoxamide and its metabolites RH-1452, RH-1455 and RH-150721
Matrix	Honey
Method Reference	S23-100692
LOQ	0.01 mg/kg (lowest validated fortification level) for all analytes
LOD	30 % of the LOQ (lowest calibration standard for all analytes)
Principle of the Analytical Procedure	Homogenisation: Stirring with spatula Propamocarb-HCl + Zoxamide: Extraction: Acetonitrile and addition of water, shaking Clean-up: none Reconstitution: Acetonitrile/0.1 % formic acid in water (1+1, v+v) Sample concentration in final extract: 0.1 g sample per mL of extract RH-1452 + RH-1455: Extraction: glycin buffer, shaking Liquid/liquid partition: ethyl acetate Clean-up: GCB SPE cartridge Reconstitution: Acetonitrile/ Water (2+8, v+v) Sample concentration in final extract: 0.2 g sample per mL of extract RH-150721: Extraction: Methanol, shaking Clean-up: none Reconstitution: 1 % acetic acid in methanol / water (4+6, v+v) Sample concentration in final extract: 0.2 g sample per mL of extract Quantification: LC-MS/MS
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions. Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.
Matrix Effects on Analyte Detection	Insignificant (< 20 %) for Zoxamide, RH-150721 Significant (> 20 %) for Propamocarb-HCl

<table>
<tr><th>Item</th><th>Activity, Result, Assessment</th></tr>
<tr><td>Calibration</td><td>Matrix-matched calibration standards
A minimum of five (5) concentration levels
Single determination
Injection of standard solutions spread over the whole acquisition batch

Propamocarb-HCl + Zoxamide:
Concentration range: 0.3 ng/mL to 3.0 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg
RH-1452 + RH-1455:
Concentration range: 0.60ng/mL to 60 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg
RH-150721:
Concentration range: 0.60ng/mL to 60 ng/mL
Corresponding mass fraction range: 0.003 mg/kg to 0.3 mg/kg

Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract
The validated range does not exceed two (2) orders of magnitude</td></tr>
<tr><td>Quantification</td><td>Linear regression with 1/x weighting
Regression residuals randomly distributed
Coefficients of correlation coefficients (R) ≥ 0.99</td></tr>
<tr><td>Accuracy
and Precision</td><td>Five (5) fortifications at 0.01 mg/kg (LOQ)
Five (5) fortifications at 0.1 mg/kg (10x LOQ)
Zoxamide and Propamocarb-HCl were fortified jointly and quantified separately.
RH-1452 and RH-1455 were fortified jointly and quantified separately.
RH-150721 was fortified and quantified separately.
Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2:
<table>
<tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr>
<tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr>
<tr><td>> 0.01 - ≤ 0.1</td><td>70 – 120</td><td>≤ 20</td></tr>
</table></td></tr>
<tr><td>Stability of Analyte(s) in Standard Solutions</td><td>Zoxamide:
Within ± 10 % for 186 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark was demonstrated in study S23-100692.
Propamocarb-HCl:
Within ± 10 % for 30 days when prepared in 1M HCl and stored at typically 1 °C to 10 °C in the dark.
Within ± 10 % for at least 7 days when prepared in 0.1 % formic acid in water/acetonitrile (1+1, v+v) and stored at typically1 °C to 10 °C in the dark was demonstrated in study S23-100692.</td></tr>
</table>

Item	Activity, Result, Assessment
	RH-1452 + RH-1455: Within ± 10 % for 186 days for RH-1452 and 214 days for RH-1455 when prepared in methanol and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 22 days when prepared in water/acetonitrile (8+2, v+v) and stored at typically1 °C to 10 °C in the dark was demonstrated in study S23-100692. RH-150721: Within ± 10 % for 172 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark. Within ± 10 % for at least 7 days when prepared in 0.1 % acetic acid in methanol/ water (4+6, v+v) and stored at typically1 °C to 10 °C in the dark. was demonstrated in study S23-100692.
Stability of Analyte(s) in Sample Extracts	Zoxamide, Promacarb-HCl, RH-1452 and RH-1455: Recoveries within 70 % - 120 % in matrix extract for 7 days when stored at typically 1 °C to 10 °C in the dark was demonstrated in study S23-100692. RH-150721: Recoveries within 70 % - 120 % in matrix extract for 8 days when stored at typically 1 °C to 10 °C in the dark was demonstrated in study S23-100692.
Independent Laboratory Validation	Primary validation and independent laboratory validation were carried out at different locations and by different study personnel. No addition or modification to the original method other than optimisation of instrumental parameters was done. No communication with the method developers or others familiar with the method was necessary to carry out the analysis.
Conclusion	The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for monitoring.

A 2.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

A 2.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted

A 2.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

Comments of zRMS:

LC-MS/MS determination was conducted with evaluation of two mass transitions (*m/z* 336→187 and *m/z* 336→159 for zoxamide and *m/z* 219→175 and *m/z* 219→147 for RH-1452). Due to enhanced sensitivity mass transition *m/z* 336→187 (zoxamide) and 219→175 (RH-1452) is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

Matrix effects were ≥ ± 20 % and deemed to be significant for RH-1452 in both matrices.

Matrix effects were < ± 20 % and deemed to be insignificant for zoxamide in both matrices. Therefore, solvent standards were used for quantification throughout the study.

The LOQ of 0.01 mg/kg was confirmed for zoxamide and RH-1452 in bovine (liver) and porcine (urine).

The following recoveries were obtained:

Zoxamide							
Mass Transition	**Fortification Level (mg/kg)**	**Recovery (%)**	**Mean Recovery (%)**	**Rel. Std. Dev. (%)**	**Replicates**	**Overall Mean Recovery (%)**	**Overall Rel. Std. Dev. (%)**
Bovine (liver)							
m/z 336→187*	0.01	89, 87, 83, 79, 79	84	5.6	5	84	4.8
	0.1	83, 83, 81, 90, 86	85	4.3	5		
m/z 336→159	0.01	91, 85, 84, 76, 78	83	7.2	5	84	5.6
	0.1	86, 85, 79, 88, 87	85	3.9	5		
Porcine (urine)							
m/z 336→187*	0.01	103, 104, 103, 107, 108	105	2.3	5	105	2.3
m/z 336→159	0.01	102, 103, 103, 110, 104	105	3.1	5	105	3.1
RH-1452							
Bovine (liver)							
m/z 219→175*	0.01	89, 90, 84, 78, 76	83	7.3	5	86	7.0
	0.1	89, 94, 90, 91, 81	89	5.6	5		
m/z 221→147	0.01	94, 86, 81, 71, 72	81	12	5	85	10
	0.1	90, 93, 89, 93, 81	89	5.5	5		
Porcine (urine)							
m/z 219→175*	0.01	98, 110, 115, 95, 92	102	9.7	5	102	10
m/z 221→147	0.01	90, 95, 95, 102, 106	98	6.6	5	98	6.6

No observable peak was detected in any control sample extract

Recoveries are without any blank correction

* Proposed to be used for quantification

The method was successfully validated for the determination of zoxamide and RH-1452 in bovine (liver) and porcine (urine) from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg (for bovine (liver) only) according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reference: KCA 4.2

Report	Validation of an Analytical Method for Determination of Zoxamide in Body Fluids and Animal Tissues, Gustloff, C., 2023, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100691
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In brief, for zoxamide samples were extracted with acetonitrile and if necessary, after addition of water. The ratio was 10 mL of extraction solvent per 5.0 g of bovine (liver) or 5 g of porcine (urine). Quantification was performed by use of LC-MS/MS.
In brief, for RH-1452 samples were extracted with glycine buffer. Liquid-liquid partition was performed twice with acetonitrile. Clean-up of the extract was performed with an ENVI-Carb SPE cartridge. Quantification was performed by use of LC-MS/MS.

Results and discussions

Item	**Activity, Result, Assessment**
Study Scope	Validation of an analytical method according to guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analytes	Zoxamide and RH-1452
Matrices	Body fluids (urine) and animal tissues (liver)
Method Reference	S21-07039 (GLC-2110V) [1] and S21-07040 (GLC-2111V) [2]
LOQ	0.01 mg/kg (lowest validated fortification level)
LOD	30 % of the LOQ (lowest calibration standard)
Principle of the Analytical Procedure	Homogenisation: Dry ice, cutter for animal tissues (liver), Shaking for body fluids (urine) **Zoxamide:** Extraction: Acetonitrile and addition of water, high-speed homogeniser (Ratio: 10 mL of extraction solvent per g of matrix) Clean-up: Dispersive SPE with primary/secondary amine (PSA) and C18 for Bovine (liver) only Concentration step: nitrogen stream Reconstitution: Acetonitrile/0.1 % formic acid (1+1, v+v) Sample concentration in final extract: 0.1 g sample per mL of extract Quantification: LC-MS/MS **RH-1452:** Extraction: glycine buffer, high-speed homogeniser for bovine (liver), shaking for porcine (urine) (Ratio: 25 mL of extraction solvent per g of matrix) Liquid-liquid-partition: two times with ethyl acetate Clean-up: ENVI-Carb cartridge Concentration step: nitrogen stream Reconstitution: Acetonitrile/water (2+8, v+v) Sample concentration in final extract: 0.2 g sample per mL of extract Quantification: LC-MS/MS

<table>
<tr><th>Item</th><th>Activity, Result, Assessment</th></tr>
<tr><td>Selectivity and Specificity</td><td>Demonstrated by validation of two (2) mass transitions
Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.</td></tr>
<tr><td>Matrix Effects on Analyte Detection</td><td>Significant (≥ 20 %) for RH-1452 in bovine (liver) and porcine (urine).
Insignificant (< 20 %) for zoxamide in bovine (liver) and porcine (urine).</td></tr>
<tr><td>Calibration</td><td>Matrix-matched calibration standards
A minimum of five (5) concentration levels
Single determination
Injection of standard solutions spread over the whole acquisition batch
Concentration range: 0.30 ng/mL to 30 ng/mL for zoxamide and 0.60 ng/mL to 60 ng/mL for RH-1452
Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg
Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract
The validated range does not exceed two (2) orders of magnitude</td></tr>
<tr><td>Quantification</td><td>Linear regression with 1/x weighting
Regression residuals randomly distributed
Coefficients of determination (R^2) ≥ 0.99</td></tr>
<tr><td>Accuracy and Precision</td><td>Five (5) fortifications at 0.01 mg/kg (LOQ)
Five (5) fortifications at 0.1 mg/kg (10x LOQ) for bovine (liver).
Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2:
<table>
<tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr>
<tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr>
<tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr>
</table></td></tr>
<tr><td>Stability of Analyte(s) in Standard Solutions</td><td>Within ± 10 % for at least 13 days when prepared in acetonitrile/0.1 % formic acid (1+1, v+v) and stored at typically 1 °C to 10 °C in the dark for zoxamide as proven in study S21-07039 (GLC-2110V) [1].
Within ± 10 % for at least 11 days when prepared in water/acetonitrile (8+2, v+v) and stored at typically 1 °C to 10 °C in the dark for RH-1452 as proven in study S21-07040 (GLC-2111V) [2].</td></tr>
<tr><td>Stability of Analytes in Sample Extracts</td><td>Recoveries within 70 % - 120 % in all matrix extracts for at least 7 days when stored at typically 1 °C to 10 °C in the dark.</td></tr>
</table>

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

A 2.1.2.7 A.2.A.9 Other Studies/ Information

No new or additional studies have been submitted.