REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: GWN-10616

Chemical active substances:

Zoxamide, 60 g/L

Potassium phosphonates, 755 g/L

Phosphonic acid equivalents, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: XXXX

Submission date: 31/10/2023

zRMS evaluation date: 07/2024

MS Finalisation date: 11/2024

Version history

When	What
July 2024	Initial RR
November 2024	RR Revision after commneting.

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5 Analytical methods

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are available for the active substances 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.
The applicant's dRR text was not rewritten by the zRMS. In the resulting RR all zRMS' comments /corrections/add-ons were placed on the grey background.
A part of zoxamide related studies presented in the Appendix 2 have been already submitted in May 2021 by XXXX. and its affiliates to the RMS Latvia and finalised in September 2023 as updated set of zoxamide data after the renewal. All these studies' summaries had to be accompanied here by the zRMS for the formal clarity with the original RMS Latvia assessment conclusions (grey boxes' content) taken from Circa from part B section 5 core assessments of the applicant products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG and GOW F113. The relevant list of studies was transfered to the list of studies "relied on but already evaluated". For all details, please see the Appendix 1 and 2.
In the context of the authorisation request noticed data gaps are: ~~None~~
Data gap: the applicant to complete the missing method for phosphonic acid residues in honey in post registration.

Commodity/crop	Supported/ Not supported
Grapevine (table and wine)	Supported
Pome fruit	Supported
Potato HONEY	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)

Comments of zRMS:	Accepted. The method is validated and can be used for analysing zoxamide and potassium phosphonate in plant protection product.

An overview on the acceptable methods and possible data gaps for analysis of Zoxamide and potassium phosphonate in plant protection product is provided as follows:

Reference:	KCP 5.1.1/01
Report:	PHYSICAL-CHEMICAL CHARACTERIZATION AND ACCELERATED

	STORAGE STABILITY (2 WEEKS/54±2°C) OF TEST ITEM GOW F716, Aversa, S., 2017, report No. BT165/17, Doc. No. 245-001
Guideline(s):	OECD No. 114, OECD No. 115, OECD No. 109, SANCO/3030/99 rev. 4, CIPAC MT 47.3, CIPAC MT 75.3, CIPAC MT 191, CIPAC MT 160, CIPAC MT 185, CIPAC MT 148.1, CIPAC MT 184, CIPAC MT 39.3, EC Reg. 440/2008 method A.5, EC Reg. 440/2008 method A.3
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples of the formulation were diluted with acetonitrile and analysed by HPLC-DAD for determination of Zoxamide. For analysis of Phosphonic acid, samples were diluted with water and analysed by HPLC-MS/MS.

Chromatographic conditions - Zoxamide

System:	HPLC-DAD
Column:	AGILENT Zorbax Eclipse Plus C18 RR 4.6 x 100 mm 3.5 µm
Mobile phase (isocratic):	Acetonitrile/Water : 65:35
Detection wavelength:	210 nm
Retention time:	Approx. 4 minutes

Chromatographic conditions – Phosphonic acid

System:	HPLC-MS/MS
Column:	PHENOMENEX Gemini 3 µm NX-C18 110 Å 150 x 3.0 mm
Mobile phase (isocratic):	A: 89.5 % water + 10 % Methanol + 0.5 % formic acid B: Acetonitrile Ratio A/B = 90:10
Monitored ions:	81 > 79 and 81 > 63
Retention time:	Approx. 1.8 minutes

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances Zoxamide and potassium phosphonate (as phosphonic acid) in plant protection product GWN-10616

	Zoxamide	**Potassium phosphonates***
Author(s), year	Aversa S. (2017), Report No. BT 165/17, Doc. No. 245-001	Aversa S. (2017), Report No. BT 165/17, Doc. No. 245-001
Principle of method	HPLC-DAD	HPLC-MS/MS
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	0.498 – 14.94 mg/L (0.249 – 7.47% w/w) y = 35.9023 x – 2.0326 n = 5 r = 0.9999	1.487 – 19.83 mg/L (7.4 – 99.2% w/w) Transition: 81>79 y = 1510.4985 x – 305.1174 n = 5 r = 1
Precision – Repeatability Mean n = 5 (%RSD)	Mean content: 4.49% w/w RSD = 0.67% Max RSD according to Horwitz: 2.14% Horrat value < 1	Transition: 81>79 Mean content: 36.56% w/w RSD = 1.10% Max RSD according to Horwitz: 1.56% Horrat value < 1

	Zoxamide	Potassium phosphonates*
Accuracy **n = 5** **(% Recovery)**	Recovery at 2% w/w: 102.76% (n = 5, RSD = 0.21% Recovery at 5.9% w/w: 97.44% (n = 5, RSD = 0.27%)	Transition: 81>79 Recovery at 30% w/w: 100.94% (n = 5, RSD = 0.57% Recovery at 40% w/w: 100.68% (n = 5, RSD = 0.81%)
Interference/ Specificity	No interferences < 3% were observed	No interferences < 3% were observed
Comment	-	-

* Potassium phosphonate was determined as phosphonic acid

Conclusion

The method is valid and acceptable according to SANCO/3030/99 rev. 5 for the determination of Zoxamide and potassium phosphonate (as phosphonic acid) in the formulation.

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

The plant protection product contains no impurities which are of toxicological, eco-toxicological or environmental concern. Therefore, an analytical methodology for their determination is not required.

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

Under current EU legislation methods on formulants are only required if they are defined as relevant with respect to toxicity. Based on the composition of the plant protection product, the development of respective analytical methods is not required. No particular substances of toxicological or eco-toxicological concerns are used as formulants.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There is no CIPAC method available for Zoxamide and for potassium phosphonates.

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 for the generation of pre-authorization 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-authorization 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**
Apple (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2021, report No. GLP-STUDY-21-53, Doc. No. 432-003
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2021, report No. GLP-STUDY-21-53, Doc. No. 432-003
Grape (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Sala, A., 2022, report No. GLP-STUDY-21-101, Doc. No. 432-006
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Sala, A., 2022, report No. GLP-STUDY-21-101, Doc. No. 432-006
Potato (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2022, report No. GLP-STUDY-21-50, Doc. No. 432-016
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2022, report No. GLP-STUDY-21-50, Doc. No. 432-016
Potato (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
Water (Ecotoxicity)	Primary	0.2492 g/L Zoxamide 2.1438g/L Phosphonic acid	HPLC-MS/MS	Fifi, A.P., 2021, report No. BT233/21, Doc. No. 435-001
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Sugar solution (Ecotoxicity)	Primary	0.1018 g/kg Zoxamide 0.8373 g/kg Phosphonic acid	HPLC-MS/MS	Fifi, A.P., 2021, report No. BT234/21, Doc. No. 437-001
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Sugar solution and water (Ecotoxicity)	Primary	1.085 µg/L in water 5.43 mg/kg in sugar feeding solution	HPLC-MS/MS	Colli, M., 2021, report No. BT147/17, Doc. No. 832-002
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Aquatic media (*Lemna*) (Ecotoxicity)	Primary Confirmatory	0.168 µg/L	HPLC-MS/MS	Juckeland, 2020 Report no. 18 48 ALE 0005*

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**
Bumblebee (Ecotoxicity)	Primary Confirmatory	50 g/L for contact test 5 g/L for oral test	HPLC-DAD	Amsel, 2018 Report no. 17 48 BBA 0017*
Honey bee royal jelly (Ecotoxicity)	Primary Confirmatory	0.5 µg/g	HPLC-MS/MS	Picard, 2018 Report no. 12791.6307*
Soil (Ecotoxicity)	Primary Confirmatory	0.05 mg/kg	LC-MS/MS	Joß, 2013 Report no. P3051G EU agreed method (RAR 2017) Method applied in: Friedrich, 2020 Report no. 17 48 TEC 0009*; Schulz, 2020 Report no. 18 48 FEW 0001*; Schulz, 2021 Report no.19 48 FEW 0002*; Parsons, 2020 Report no. GOW-17-13*; Parsons, 2020 Report no.GOW-17-14*

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**
Component analysed: RH-163353				
Aquatic test media (Ecotoxicity)	Primary	0.001 mg/L (Fish) 0.1 mg/L (*Daphnia*, alga, mysid)	LC-TOF/MS	Goodband, 2020 Report no. 3202385* Jarrom, 2020; Report no. 3202386*; Report no. 3202387*; Report no. 3202388*
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Component(s) analysed: RH-163353 (R, S, sum)				
Soil (Ecotoxicity)	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS	Gray, 2021 Report no. 3202389*; Report no. 3202390*; Report no. 3202391*
Component analysed: RH-127450				
Aquatic media (Ecotoxicity)	Primary Confirmatory	0.01 mg/L (alga, mysid) and 0.001 mg/L (fish)	LC-TOF/MS LC-TQMS	Goodband, 2020 Report no. 3202373*; Hugill, 2020 Report no. 3202374*; Hugill, 2020 Report no. 3202375*
Soil (Ecotoxicity)	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS	Gray, 2021 Report no. 3202376*
Component(s) analysed: RH-139432				
Aquatic media (Ecotoxicity)	Primary Confirmatory	0.1 mg/L (mysid)	LC-TOF/MS LC-TQMS	Hugill, 2020 Report no. 3202398*
Component(s) analysed: RH-24549				
Aquatic media (Ecotoxicity)	Primary Confirmatory	0.1 mg/L (mysid)	LC-TOF/MS LC-TQMS	Hugill, 2020 Report no. 3202394*
Soil (Ecotoxicity)	Primary Confirmatory	0.016 mg/kg	LC-TOF/MS LC-TQMS	Gray, 2021 Report no. 3202395*
Component(s) analysed: RH-117,281				
Salt water (fish) (Ecotoxicity)	Primary	0.01 mg/L	HPLC-UV	Drottar *et al.*, 1998 Report no. 97RC-0078* & Milligan *et al.*, 2020 Report No. 129A-143A*

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**
Component(s) analysed: RH-141455				
Aqueous buffer samples (Residues)	Primary	1 mg/L	HPLC-MS/MS	Longhi, D., 2019, report No. BPL-STUDY-19-000009, Doc. No. 638-009*
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Aquatic test media (Ecotoxicity)	Primary Confirmatory	0.25 mg/L (mysid) 0.1 mg/L (Daphnia, fish)	LC-TOF/MS	Goodband, 2020 Report no. 3202716* Hugill, 2019 Report no. 3202380* Hugill, 2020 Report no. 3202381*
Soil (Ecotoxicity)	Primary Confirmatory	0.2 mg/kg	LC-TOF/MS LC-FT/MS	Gray, 2021 Report no. 3202382*; Report no. 3202383*
Rat feed (Toxicity)	Primary	0.005 mg/mL	HPLC-UV	Nagarajan, 2019 Report no. U-19069*
Blood plasma (Toxicity)	Primary Confirmatory	0.104 µg/mL	LC-MS/MS	XXXX, 2019 Report no. U-19044 * XXXX, 2020 Report no. U-19102*; Report no. U-19071*
Potato (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017

* Studies have already been provided to the RMS Latvia and are only provided for completeness

Component of residue definition: RH-141452				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Grape (Residues)	Primary	0.01 mg/kg	HPLC-HRMS/MS	Sala, A., 2022, report No. GLP-STUDY-21-102, Doc. No. 432-007
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Apple (Residues)	Primary	0.01 mg/kg	HPLC-HRMS/MS	Longhi, D., 2021, report No. GLP-STUDY-21-54, Doc. No. 432-005
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev.
Potato (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
	Confirmatory (if required)	0.01 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
Aqueous buffer samples (Residues)	Primary	1 mg/L	HPLC-MS/MS	Longhi, D., 2019, report No. BPL-STUDY-18-000092, Doc. No. 638-008*
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2

* Study has already been provided to the RMS Latvia and is only provided for completeness

Component of residue definition: RH-150721				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Grape fruits Grape juice Wine Raisins Tomato fruits Canned tomatoes Potato tubers Potato flakes Fried potatoes Cucumber fruits Onion bulbs (Residues)	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085*
Rat feed (Toxicity)	Primary	0.091 mg/mL	HPLC-UV	Nagarajan, 2020 Report no. U-19162*

* Study has already been provided to the RMS Latvia and is only provided for completeness

Component of residue definition: phosphonic acid and their salts, expressed as phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Dermal absorption matrices (Toxicity)	Primary	receptor fluid and receptor wash: 2.0 ng/mL tape strips: 3.0 ng/mL skin: 3.0 ng/mL	HPLC-MS/MS	Maire, F., 2022, report No. 20352080, Doc. No. 437-002
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Dislodging solution (Toxicity)	Primary	0.2 µg/L Zoxamide 1 µg/L Phosphonic acid	HPLC-MS/MS	Palau, I., 2023, Report No. ACI22-009
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Grape (Residues)	Primary	1 mg/kg	HPLC-MS/MS	Longhi, D., 2021, report No. GLP-STUDY-20-38, Doc. No. 432-010
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Grape (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Sala, A., 2022, report No. GLP-STUDY-21-103, Doc. No. 432-008
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Apple (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2021, report No. GLP-STUDY-21-55, Doc. No. 432-004
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Apple (Residues)	Primary	1 mg/kg	HPLC-MS/MS	Longhi, D., 2020, report No. BPL-STUDY-19-000111, Doc. No. 432-014
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Potato (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Longhi, D., 2022, report No. GLP-STUDY-21-52, Doc. No. 432-015
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2

Component of residue definition: phosphonic acid and their salts, expressed as phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Potato tuber Crisps Starch Wet peel, Microwaved/boiled potatoes Baked potatoes, Process paste, Flakes, Potato protein, Fried potatoes, Chips/ french fries, Canned potatoes, Ensiled potatoes Dried pulp (Residues)	Primary	Tuber 0.02 mg/kg Crisps 0.05 mg/kg Starch 0.02 mg/kg Wet peel 0.02 mg/kg Microwaved/ Boiled Potatoes 0.02 mg/kg Baked Potatoes 0.02 mg/kg Process Waste 0.04 mg/kg Flakes 0.06 mg/kg Potato Protein 0.02 mg/kg Fried Potatoes 0.02 mg/kg Chips/ French Fries 0.02 mg/kg Canned potatoes 0.05 mg/kg Ensiled potatoes 0.02 mg/kg Dried pulp 0.02 mg/kg	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017
	Confirmatory (if required)	Tuber 0.02 mg/kg Crisps 0.05 mg/kg Starch 0.02 mg/kg Wet peel 0.02 mg/kg Microwaved/ Boiled Potatoes 0.02 mg/kg Baked Potatoes 0.02 mg/kg Process Waste 0.04 mg/kg Flakes 0.06 mg/kg Potato Protein 0.02 mg/kg Fried Potatoes 0.02 mg/kg Chips/ French Fries 0.02 mg/kg Canned potatoes	HPLC-MS/MS	Link, T. 2023. Report No. IF23-06197316, Doc. No. 432-017

Component of residue definition: phosphonic acid and their salts, expressed as phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
		0.05 mg/kg Ensiled potatoes 0.02 mg/kg Dried pulp 0.02 mg/kg		
Water (Ecotoxicity)	Primary	0.2492 g/L Zoxamide 2.1438g/L Phosphonic acid	HPLC-MS/MS	Fifi, A.P., 2021, report No. BT233/21, Doc. No. 435-001
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Sugar solution (Ecotoxicity)	Primary	0.1018 g/kg Zoxamide 0.8373 g/kg Phosphonic acid	HPLC-MS/MS	Fifi, A.P., 2021, report No. BT234/21, Doc. No. 437-001
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2
Sugar solution and water (Ecotoxicity)	Primary	834.46 mg/L in water 43.37 mg/kg in sugar feeding solution	HPLC-MS/MS	Colli, M., 2021, report No. BT147/17, Doc. No. 832-002
	Confirmatory (if required)	-	-	Not required according to SANTE/2020/12830 rev. 2

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 not identical. Residue definitions which were not finalised during a.s. renewal (EFSA Journal 2017;15(9):4980) were agreed during the dRR evaluation by RMS Latvia and by EFSA for Art. 12 evaluation.

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	0.02 mg/kg	Reg. (EU) 2017/171
Plant, high acid content		5 mg/kg	Reg. (EU) 2017/171
Plant, high protein/high starch content (dry commodities)		-	Not included in intended uses.
Plant, high oil content		-	Not included in intended uses.
Plant, difficult matrices (hops, spices, tea)		-	Not included in intended uses.
Muscle	Not relevant (tentative)	0.01 mg/kg	Reg. (EU) 2017/171
Milk		0.01 mg/L	Reg. (EU) 2017/171
Eggs		0.01 mg/kg	Reg. (EU) 2017/171
Fat		0.01 mg/kg	Reg. (EU) 2017/171
Liver, kidney		0.01 mg/kg	Reg. (EU) 2017/171
Soil (Ecotoxicology)	Zoxamide	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Zoxamide	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Zoxamide	0.00348 mg/L (*Oncorhynchus mykiss*)	EFSA Journal 2017;15(9):4980
Air	Zoxamide	90 µg/m^3	$AOEL_{syst.}$: 0.3 mg/kg bw/d
Tissue (meat or liver)	Zoxamide	0.01 mg/kg	SANTE/2020/12830 rev. 2
Body fluids		0.01 mg/L	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**	**Author(s), year / missing / EU agreed**
High water content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 Report no. S12-03949 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 Report no. P3114G EU agreed method (RAR 2017)
	ILV	0.01 mg/kg	LC-MS/MS (QuEChERS)	Schlewitz, 2014 Report no. R B4023 EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G 10186-01-VMPL New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS	Longhi, D., 2021, report No. GLP-STUDY-21-53, Doc. No. 432-003
	ILV	0.01 mg/kg	LC-MS/MS	López Benet, F., 2023, report No. 435-22, Doc. No. 432-001
High acidic content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 Report no. S12-03949 EU agreed method (RAR 2017)
	ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 Report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G 10186-01-VMPL New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 New method submitted to RMS Latvia

Component of residue definition: Zoxamide				
Matrix type	**Method type**	**Method LOQ**	**Principle of method**	**Author(s), year / missing / EU agreed**
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS	Sala, A., 2022, report No. GLP-STUDY-21-101, Doc. No. 432-006
	ILV	0.01 mg/kg	LC-MS/MS	López Benet, F., 2023, report No. 435-22, Doc. No. 432-001
High oil content	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 Report no. S12-03949 EU agreed method (RAR 2017)
	ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter, 2014 Report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G 10186-01-VMPL New method
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala, 2020 Report no. BPL-STUDY-18-000085 New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS	Longhi, D., 2022, report No. LBN-0001-2022, Doc. No. 432-002
	ILV	0.01 mg/kg	LC-MS/MS	López Benet, F., 2023, report No. 435-22, Doc. No. 432-001
High protein/high sugar/high starch content (dry)	Primary Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Weber, 2012 Report no. S12-03949 EU agreed method (RAR 2017)
	ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Richter , 2014 Report no. P3114G EU agreed method (RAR 2017)
	Primary/ILV Confirmatory	0.01 mg/kg	LC-MS/MS (QuEChERS)	Witte, 2020 Report no. 18G 10186-01-VMPL New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS (QuEChERS)	Sala , 2020 Report no. BPL-STUDY-18-000085 New method submitted to RMS Latvia
	Primary Confirmatoy	0.01 mg/kg	LC-MS/MS	Longhi, D., 2022, report No. LBN-0001-2022, Doc. No. 432-002
	ILV	0.01 mg/kg	LC-MS/MS	López Benet, F., 2023, report No. 435-22, Doc. No. 432-001

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:	Evaluated in the RAR (2017) and addressed in the dRR sent to RMS Latvia RAR (2017): Hein, W. (2014), Report No. AS362 dRR Latvia: Sala, A. (2020), Report No. BPL-Study 18000085
Not required, because:	-

For the detailed evaluation of (additional) studies on extraction efficiency, it is referred to Appendix 2.

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

No new data. / No relevant for this submission.

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

An overview on the acceptable methods and possible data gaps for analysis of Zoxamide in soil is given in the following tables. 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	Joß, 2013 report no. P3051G EU agreed method (RAR, 2017)
Confirmatory	0.05 mg/kg	LC-MS/MS	Joß, 2013 report no. P3051G EU agreed method (RAR, 2017)

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	Joß, 2013 report no. P3050G EU agreed method (RAR, 2017)
	ILV	0.1 µg/L	LC-MS/MS	Schlewitz, 2014 report no. RB4049 EU agreed method (RAR, 2017)
	Confirmatory	0.1 µg/L	LC-MS/MS	Joß, 2013 report no. P3050G EU agreed method (RAR, 2017)
Surface water	Primary	0.1 µg/L	LC-MS/MS	Joß, 2013 report no. P3050G EU agreed method (RAR, 2017)
	Confirmatory	0.1 µg/L	LC-MS/MS	Joß, 2013 report no. P3050G EU agreed method (RAR, 2017)

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	HPLC-MS/MS	Miller, C. (2013), Report No. FRK0048 EU agreed method (RAR, 2017)
Confirmatory	-	-	Not required according to SANTE/2020/12830 rev. 2

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)

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

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

Component of residue definition: Zoxamide			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.01 mg/L (body fluids; plasma and urine)	LC-MS/MS	Longhi, D. (2022), Report No. LBN-0002-2022
Confirmatory	0.01 mg/kg (tissues; bovine meat)	LC-MS/MS	Longhi, D. (2022), Report No. LBN-0002-2022

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

None

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

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

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

Table 5.3-8: 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	Phosphonic acid and its salts expressed as phosphonic acid	200 mg/kg (potato) [1]	Regulation (EU) 2022/1324
		150 mg/kg (apple) [1]	Regulation (EU) 2022/1324
Plant, high acid content		100 mg/kg (table grape) [1]	Regulation (EU) 2022/1324
Plant, high protein/high starch content (dry commodities)		150 mg/kg (not part of this application) [1]	Regulation (EU) 2022/1324
Plant, high oil content		2 mg/kg (not part of this application) [1]	Regulation (EU) 2022/1324
Muscle	Not required	Not required	EFSA Journal 2012;10(12):2963 for

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
			Potassium phosphonates
Milk		Not required	EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Eggs		Not required	EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Fat		Not required	EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Liver, kidney		Not required	EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Soil (Ecotoxicology)	Phosphonic acid and its salts expressed as phosphonic acid	0.05 mg/kg	Common limit
Drinking water (Human toxicology)	Phosphonic acid and its salts expressed as phosphonic acid	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Phosphonic acid and its salts expressed as phosphonic acid	> 11800 µg/L	LC_{50} *O. mykiss* (96h) EC_{50} *Daphnia magna* (48h) EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Air	Phosphonic acid and its salts expressed as phosphonic acid	1500 µg/m^3	AOEL: 5 mg/kg bw/d EFSA Journal 2012;10(12):2963 for Potassium phosphonates
Tissue (meat or liver)	Phosphonic acid and its salts expressed as phosphonic acid	0.01 mg/kg	SANTE/2020/12830 rev. 2
Body fluids		0.01 mg/L	SANTE/2020/12830 rev. 2

[1] MRL for Fosetyl-Al according to Regulation (EU) 2022/1324.

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

An overview on the acceptable methods and possible data gaps for analysis of Phosphonic acid in plant matrices is given in the following tables. For the detailed evaluation of new/additional studies it is referred to Appendix 2. Referring to the data gap mentioned in the EFSA conclusion (EFSA Journal 2012;10(12):2963) “Residue monitoring methods in soil and water or additional validation data for the proposed methods using a different methylation agent (relevant for the representative use evaluated; submission date proposed by the applicant unknown; see section 1)”, this point is addressed in the letter of access to phosphonic acid data from Luxembourg.

Table 5.3-9: 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: Phosphonic acid and its salts expressed as Phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed
	ILV	0.5 mg/kg	HPLC-MS/MS	Mende, P. (2011), Report No. S11-03203 / EU agreed
	Confirmatory (if required)	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed
High acid content	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-09/01419440 / EU agreed
	ILV	0.5 mg/kg	HPLC-MS/MS	Mende, P. (2011), Report No. S11-03203 / EU agreed
	Confirmatory (if required)	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-09/01419440 / EU agreed
High oil content	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed
	ILV	-	-	Covered by ILV by Mende, P. (2011), Report No. S11-03203
	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed
High protein/high starch content (dry)	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed
	ILV	-	-	Covered by ILV by Mende, P. (2011), Report No. S11-03203
	Primary	0.5 mg/kg	HPLC-MS/MS	Toledo, F. (2011), Report-no. IF-10/01711965 / EU agreed

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 5.3-10: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	-
Not required, because:	Since no metabolism studies for phosphonic acid are available, an assessment of extraction efficiency as outlined in SANTE/2017/10632 rev. 5 cannot be made. Considering that phosphonic acid and its salt is an inorganic compound with high solubility in water and polar solvents, it can be assumend that extraction with polar solvents has sufficient extraction efficiency.

For the detailed evaluation of (additional) studies on extraction efficiency, it is referred to Appendix 2.

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

Analytical methods for the determination of residues of potassium phosphonate in animal matrices are not required.

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

An overview on the acceptable methods and possible data gaps for analysis of Phosphonic acid and its salts expressed as Phosphonic acid in soil is given in the following tables. For the detailed evaluation of new/additional studies it is referred to Appendix 2 and the letter of access.

Table 5.3-11: Validated methods for soil (if appropriate)

Component of residue definition: Phosphonic acid and its salts expressed as phosphonic acid			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.05 mg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVS / EU agreed
Confirmatory	0.05 mg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVS / EU agreed

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

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

An overview on the acceptable methods and possible data gaps for analysis of Phosphonic acid and its salts expressed as Phosphonic acid 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 and the letter of access.

Table 5.3-12: Validated methods for water (if appropriate)

Component of residue definition: Phosphonic acid and its salts expressed as phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.5 µg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVW / EU agreed
	ILV	-	-	Letter of access is available.
	Confirmatory	0.5 µg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVW / EU agreed

Component of residue definition: Phosphonic acid and its salts expressed as phosphonic acid				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Surface water	Primary	0.5 µg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVW / EU agreed
	Confirmatory	0.5 µg/kg	GC-NPD / GC-MS	Hamberger, R. (2006), Report No. 20061235/01-RVW / EU agreed

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

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

An overview on the acceptable methods and possible data gaps for analysis of Phosphonic acid and its salts expressed as Phosphonic acid in air is given in the following tables. For the detailed evaluation of new/additional studies please refer to Appendix 2.

Table 5.3-13: Validated methods for air (if appropriate)

Component of residue definition: Phosphonic acid and its salts expressed as phosphonic acid			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
~~Primary~~	-	-	~~Letter of access is available.~~
~~Confirmatory~~	-	-	~~Letter of access is available.~~

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

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

An overview on the acceptable methods and possible data gaps for analysis of Phosphonic acid and its salts expressed as Phosphonic acid in body fluids and tissues is given in the following table. For the detailed evaluation of new/additional studies it is referred to Appendix 2.

Table 5.3-14: Methods for body fluids and tissues (if appropriate)

Component of residue definition: Phosphonic acid and its salts expressed as phosphonic acid			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
~~Primary~~	-	-	~~Letter of access is available.~~
~~Confirmatory~~	-	-	~~Letter of access is available.~~

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

5.3.3.8 Other studies/ information

Not required

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	Verte-brate study Y/N	Owner
KCP 5.1.1/01	Aversa, S.	2017	PHYSICAL-CHEMICAL CHARACTERIZATION AND ACCELERATED STORAGE STABILITY (2 WEEKS/54±2°C) OF TEST ITEM GOW F716 BT165/17 (245-001) BIOTECNOLOGIE BT S.r.l., Todi, Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (c)/01	Maire, F.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTITATIVE ANALYSIS OF PHOSPHOROUS ACID TO SUPPORT THE IN VITRO DERMAL ABSORPTION STUDY 20352080 (437-002) Charles River Laboratories Den Bosch BV, 's-Hertogenbosch, The Netherlands GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/01	Longhi, D.	2021	ANALYTICAL METHOD VALIDATION TO QUANTIFY PHOSPHONIC ACID RESIDUES IN GRAPE BUNCHES (HIGH ACID CONTENT MATRIX) AND WINE GLP-STUDY-20-38 (432-010) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/02	Sala, A.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN GRAPES GLP-STUDY-21-103 (432-008) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/03	Longhi, D.	2021	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN APPLES RAC AND PROCESSED COMMODITIES GLP-STUDY-21-55 (432-004) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.1.2 (e)/04	Longhi, D.	2020	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN HIGH WATER CONTENT AGRICULTURAL COMMODITIES (APPLE) BPL-STUDY-19-000111 (432-014) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/05	Longhi, D.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN POTATO GLP-STUDY-21-52 (432-015) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/06	Sala, A.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RH-141452 (TOTAL FRACTION) IN GRAPES GLP-STUDY-21-102 (432-007) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/07	Longhi, D.	2021	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RH-141452 (TOTAL FRACTION) IN APPLES GLP-STUDY-21-54 (432-005) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/08	Longhi, D.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN POTATO GLP-STUDY-21-50 (432-016) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/09	Link, T.	2023	VALIDATION OF ANALYTICAL METHODS FOR DETERMINATION OF GWN-8030, MDI-0043, MDI-0050 AND MDI-0074 IN POTATO MATRICES IF23-06197316 (432-017) SGS Institut Fresenius GmbH, Taunusstein, Germany GLP, unpublished	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.1.2 (f)/01	Fifi, A.P.	2021	VALIDATION OF THE ANALYTICAL METHODS (SANTE/2020/12830 REV.1) FOR THE DETERMINATION OF PHOSPHONIC ACID AND ZOXAMIDE IN AQUEOUS MATRIX SOLUTIONS WITH PRODUCT GWN-10616 BT233/21 (435-001) BIOTECNOLOGIE BT S.r.l., Todi, Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (f)/02	Fifi, A.P.	2021	VALIDATION OF THE ANALYTICAL METHODS (SANTE/2020/12830 REV.1) FOR THE DETERMINATION OF PHOSPHONIC ACID AND ZOXAMIDE IN SUGAR FEEDING SOLUTIONS WITH PRODUCT GWN-10616 BT234/21 (437-001) BIOTECNOLOGIE BT S.r.l., Todi, Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (f)/03	Colli, M.	2021	CHRONIC ORAL EFFECTS OF GOW F716 (GWN-10616) TO ADULT WORKER HONEYBEES APIS MELLIFERA L. 10-DAY FEEDING LABORATORY TEST BT147/17 (832-002) BIOTECNOLOGIE BT S.r.l., Todi, Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (g)/01	Fieseler, A.	2022	GWN-10392: EFFECTIVENESS OF CLEANING PROCEDURES 164781361 (247-001) Ibacon GmbH, Rossdorf, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/01	Longhi, D.	2021	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN APPLES GLP-STUDY-21-53 (432-003) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.2 (a)/02	Sala, A.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN GRAPES GLP-STUDY-21-101 (432-006) LabAnalysis s.r.l., Casanova Lonati (PV), Italy	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			GLP, unpublished		
KCP 5.2 (a)/03	Longhi, D.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTIFICATION OF ZOXAMIDE IN HIGH OIL CONTENT AND DRY PLANT MATRICES LBN-0001-2022 (432-002) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.2 (a)/04	López Benet, F.	2023	INDEPENDENT LABORATORY VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF GWN-8030 IN PLANT MATRICES 435-22 (432-001) Laboratorio de Analisis de Residuos de Plaguicidas, Castellón, Spain GLP, unpublished	N	XXXX
KCP 5.2 (d)/01	Longhi, D.	2022	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTIFICATION OF ZOXAMIDE IN BODY FLUIDS AND TISSUE LBN-0002-2022 (433-001) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.1.2 (c)/02	XXXX	2020	RH-141455: 90-day oral dietary toxicity study with toxicokinetics and 28-day recovery period in Sprague Dawley rats XXXX, Report No. U-19102 GLP Not published	Y	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.1.2 (c)/03	XXXX	2019	RH-141455: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX, Report No. U-19044 No GLP Not published	Y	XXXX
KCP 5.1.2 (c)/04	XXXX	2020	RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX, Report No. U-19071 No GLP Not published	Y	XXXX
KCP 5.1.2 (c)/05	XXXX	2020	RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX, Report No. U-19134 No GLP Not published	Y	XXXX
KCP 5.1.2 (c)/06	Nagarajan, S.	2020	Analytical method validation for the estimation of RH-150721 in rat feed by reverse phase high performance liquid chromatography Gowan Crop Protection Ltd., UK Syngene International Ltd., India, Report No. U-19162 GLP Not published	N	XXXX
KCP 5.1.2 (c)/07	Nagarajan, S.	2019	Analytical method validation for the estimation of RH-141455 in rat feed by reverse phase high performance liquid chromatography was used for the dose formulation analysis Gowan Crop Protection Ltd., UK Syngene International Ltd., India, Report No. U-19069 GLP Not published	N	XXXX
KCP 5.1.2 (e)/10	Longhi, D.	2019	RH-141452: HYDROLYSIS UNDER SIMULATED PROCESSING CONDITIONS BPL-STUDY-18-000092 (638-008) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.1.2 (e)/11	Longhi, D.	2019	RH-141455: HYDROLYSIS UNDER SIMULATED PROCESSING CONDITIONS BPL-STUDY-19-000009 (638-009)	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished		
KCP 5.1.2 (f)/04	Goodband, T.	2020	RH-163353: Fish, acute toxicity test – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202385 GLP Not published	Y	XXXX
KCP 5.1.2 (f)/05	Jarrom, R.	2020	RH-163353**:** Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202386 GLP Not published	N	XXXX
KCP 5.1.2 (f)/06	Jarrom, R.	2020	RH-163353: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202387 GLP Not published	N	XXXX
KCP 5.1.2 (f)/07	Jarrom, R.	2020	RH-163353: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202388 GLP Not published	N	XXXX
KCP 5.1.2 (f)/08	Goodband, T.	2020	RH-141455: Fish, acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202716 GLP Not published	Y	XXXX
KCP 5.1.2 (f)/09	Hugill, E.	2020	RH-141455: Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202380	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			GLP Not published		
KCP 5.1.2 (f)/10	Hugill, E.	2020	RH-141455: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202381 GLP Not published	N	XXXX
KCP 5.1.2 (f)/11	Goodband, T.	2020	RH-127450: Fish, acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202373 GLP Not published	Y	XXXX
KCP 5.1.2 (f)/12	Hugill, E.	2020	RH-127450: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202374 GLP Not published	N	XXXX
KCP 5.1.2 (f)/13	Hugill, E.	2020	RH-127450: Inhibition of growth on the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202375 GLP Not published	N	XXXX
KCP 5.1.2 (f)/14	Hugill, E.	2020	RH-139432: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK Report No. 3202398 GLP Not published	N	XXXX
KCP 5.1.2 (f)/15	Hugill, E.	2020	RH-24549: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202394	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			GLP Not published		
KCP 5.1.2 (f)/16	Juckeland, D.	2020	Effects of Zoxamide technical on *Lemna gibba* in a growth inhibition test under semi-static test conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 18 48 ALE 0005 GLP Not published	N	XXXX
KCP 5.1.2 (f)/17	Milligan, Amanda L., Martin, Kathy H., Schneider, Suzanne Z	2020	Milligan, Amanda L., Martin, Kathy H., Schneider, Suzanne Z., 2020: Final report addendum for RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) Gowan Crop Protection Ltd., UK Eurofins EAG Agroscience, LLC, USA, Report No. 129A-143A GLP Not published	N	XXXX
KCP 5.1.2 (f)/18	Drottar, K.R., Krueger, H.O.	1998	RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) Rohm & Haas Company, USA, Report No. 97RC-0078 Gowan Crop Protection Ltd., UK Wildlife International Ltd., USA, Report No. 129A-143A GLP Not published	Y	XXXX
KCP 5.1.2 (f)/19	Amsel, K.	2018	Acute toxicity of Zoxium 240 SC to the bumblebee *Bombus terrestris* L. under laboratory conditions Gowan Crop Protection Ltd. BioChem agrar, Germany, Report No. 17 48 BBA 0017 GLP Not published	N	XXXX
KCP 5.1.2 (f)/20	Picard, Ch.R.	2018	Zoxamide: Honey bee (*Apis mellifera* L.) larval toxicity, repeated exposure Exigent LLC, A Gowan Group Company, USA Smithers Viscient, USA, Report No. 12791.6307 GLP	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Not published		
KCP 5.1.2 (f)/21	Friedrich, S.	2020	Effects of Zoxium 240 SC on the reproduction of the earthworm *Eisenia andrei* in artificial soil with 5 % peat Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 17 48 TEC 0009 GLP Not published	N	XXXX
KCP 5.1.2 (f)/22	Gray, J.	2021	RH-127450: Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd, UK, Report No.3202376 GLP Not published	N	XXXX
KCP 5.1.2 (f)/23	Gray, J.	2021	RH-24549: Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No.3202395 GLP Not published	N	XXXX
KCP 5.1.2 (f)/24	Gray, J.	2021	RH-163353: Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No.3202389 GLP Not published	N	XXXX
KCP 5.1.2 (f)/25	Gray, J.	2021	RH-163353: Collembolan reproduction test in soil – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202390 GLP Not published	N	XXXX
KCP 5.1.2 (f)/26	Gray, J	2021	RH-163353: Effect on reproduction of *Hypoaspis* (*Geolaelaps*) *aculeifer* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202391	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			GLP Not published		
KCP 5.1.2 (f)/27	Schulz, L.	2020	Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK, BioChem agrar, Germany, Report No.18 48 FEW 0001 GLP Not published	N	XXXX
KCP 5.1.2 (f)/28	Schulz, L.	2021	Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 19 48 FEW 0002 GLP Not published	N	XXXX
KCP 5.1.2 (f)/29	Gray, J.	2021	RH-141455: Collembolan reproduction study – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202382 GLP Not published	N	XXXX
KCP 5.1.2 (f)/30	Gray, J.	2021	RH-141455: Effect on reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202383 GLP Not published	N	XXXX
KCP 5.1.2 (f)/31	Parsons, Ch	2020	Zoxium 240 SC - A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, Report No. GOW-17-13 GLP Not published	N	XXXX
KCP 5.1.2 (f)/32	Parsons, Ch	2020	Zoxium 240 SC – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, Report No. GOW-17-14 GLP Not published		
KCP 5.2 (a)/05	Sala, A.	2020	VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE ZOXAMIDE RESIDUES IN GRAPE, POTATO, TOMATO, CUCUMBER, AND ONION RAW AGRICULTURAL AND PROCESSED COMMODITIES BPL-STUDY-18-000085 (432-009) LabAnalysis s.r.l., Casanova Lonati (PV), Italy GLP, unpublished	N	XXXX
KCP 5.2 (a)/06	Poráczki, K.	2020	MAGNITUDE OF RESIDUES OF ZOXAMIDE IN PHACELIA (PHACELIA TANACETIFOLIA BENTH.) HONEY AFTER THREE APPLICATIONS OF GWN-9790EU UNDER SEMI-FIELD CONDITIONS IN NORTHERN AND SOUTHERN EUROPE 19 48 BTR 0003 (634-96001) BioChem agrar Labor für biologische und chemische Analytik GmbH, Gerichshain, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/07	Witte, A.	2020	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF R/S-ISOMERS OF ZOXAMIDE AND METABOLITES RH-141452 AND RH-141455 IN 4 DIFFERENT MATRICES: POTATO TUBERS (WATER CONTAINING MATRIX), POTATO FLAKES (DRY MATRIX), POTATO CHIPS (FAT CONTAINING MATRIX) AND PICKLED SILVER SKIN ONIONS (ACIDIC MATRIX 18G10186-01-VMPL (432-011) CIP Chemisches Institut Pforzheim GmbH, Pforzheim, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/08	Weber, H.	2012	VALIDATION OF AN ENFORCEMENT METHOD (“QUECHERS”) FOR THE DETERMINATION OF RESIDUES OF ZOXAMIDE IN GRAPES AND POTATOES AND THEIR PROCESSED PRODUCTS USING LC-MS/MS S12-03949 (994-04001)	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Eurofins Agroscience Services Chem GmbH, Hamburg, Germany GLP, unpublished		
KCP 5.2 (a)/09	Richter, S.	2010	VALIDATION OF THE QUECHERS MULTI-RESIDUE METHOD FOR THE DETERMINATION OF ZOXAMIDE IN VARIOUS CROP TYPES P 3114G (994-04002) PTRL Europe, Ulm, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/10	Schlewitz, P.	2014	INDEPENDENT LABORATORY VALIDATION OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF ZOXAMIDE RESIDUES IN LETTUCE R B4023 (994-04003) Anadiag, Haguenau, France GLP, unpublished	N	XXXX
KCP 5.2 (a)/11	Hein, W.	2014	EXTRACTION EFFICIENCY OF [PHENYL-UL-14C] ZOXAMIDE FROM PLANT METABOLISM SAMPLES (PEA) AS362 (994-04010) Rheinland-Pfalz AgroScience GmbH, Neustadt/Wstr., Germany GLP, unpublished	N	XXXX
KCP 5.2 (b)/01	Jooß, S.	2013	DEVELOPMENT AND VALIDATION OF A RESIDUE METHOD FOR THE DETERMINATION OF ZOXAMIDE IN SOIL P 3051 G PTRL Europe, Ulm, Germany GLP, unpublished	N	XXXX
KCP 5.2 (b)/02	Jooß, S.	2013	DEVELOPMENT AND VALIDATION OF A RESIDUE METHOD FOR THE DETERMINATION OF ZOXAMIDE INDRINKING AND IN SURFACE WATER P 3050 G PTRL Europe, Ulm, Germany GLP, unpublished	N	XXXX
KCP 5.2 (b)/03	Schlewitz, P.	2014	INDEPENDENT LAB VALIDATION OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF ZOXAMIDE RESIDUES IN DRINKING WATER B4049 (435-002)	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Anadiag, Haguenau, France GLP, unpublished		
KCP 5.2 (c)/01	Miller, C.	2014	ZOXAMIDE: VALIDATION OF METHODOLOGY FOR THE DETERMINATION OF RESIDUES IN AIR FRK0048 (994-04004) Huntingdon Life Sciences GLP, unpublished	N	XXXX
KCP 5.2 (a)/12	Toledo, F.	2011	DETERMINATION OF PHOSPHONIC ACID IN PLANT MATRICES: LETTUCE,RAPE SEED AND CEREAL GRAIN - VALIDATION OF THE METHOD - IF-10/01711965 (994-04006) SGS Institut Fresenius GmbH, Taunusstein, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/13	Toledo, F.	2010	DETERMINATION OF PHOSPHONIC ACID IN GRAPES AND GRAPE-PROCESSED FRACTIONS - VALIDATION OF THE METHOD IF-09/01419440 (994-04005) SGS Institut Fresenius GmbH, Taunusstein, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/14	Mende, P.	2011	INDEPENDENT LABORATORY VALIDATION (ILV) OF AN ANALYTICAL METHOD FOR DE-TERMINATION OF RESIDUES OF PHOSPHONIC ACID IN FOOD OF PLANT ORIGIN S11-03203 (994-04007) Eurofins Agroscience Services EcoChem GmbH, Niefern-Öschelbronn, Germany GLP, unpublished	N	XXXX
KCP 5.2 (a)/15	Laurent, M.; Chabassol, Y.	1982	Fosetyl-Al (32545 R.P.) - Metabolism in pineapples Rhone-Poulenc, Centre Nicolas Grillet, Vitry-sur-Seine, France Bayer CropScience, Report No.: R000790, Edition Number: M-159340-01-1 Date: 1982-01-20 GLP/GEP: no, unpublished	N	XXXX

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.2 (b)/04	Hamberger, R.	2006	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHO-ROUS ACID IN SOIL 20061235/01-RVS (994-04008) GLP, unpublished	N	Luxembourg Industries Ltd.
KCP 5.2 (b)/05	Hamberger, R.	2006	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHO-ROUS ACID IN WATER 20061235/01-RVW (994-04009) GLP, unpublished	N	Luxembourg Industries Ltd.

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for Zoxamide and phosphonic acid

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

Methods used in toxicity studies

Determination of phosphonic acid in support of a dermal absorption study

Comments of zRMS:	The validation has been accepted. UPLC-MS/MS method was applied for the quantitative analysis of the test material phosphorous acid. It was successfully validated in 5 matrices tested.

Reference:	KCP 5.1.2 (c)/01
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTITATIVE ANALYSIS OF PHOSPHOROUS ACID TO SUPPORT THE IN VITRO DERMAL ABSORPTION STUDY, Maire, F., 2022, report No. 20352080, Doc. No. 437-002
Guideline(s):	SANTE/2020/12830 Rev.1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were mixed with 2% hexylamine/0.4% formic acid in water (v/v/v) and internal standard solutions. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Acquity UPLC BEH 18 100 mm x 2.1 mm ID, 1.7 µm particle size
Mobile phase	A: 0.1% Hexylamine + 0.02% formic acid in water B: 0.1% Hexylamine + 0.02% formic acid in acetonitrile
Monitored ions	Phosphonic acid: 81.0 > 79.0 Phosphonic acid-^{18}O (internal standard): 87.0 > 85.0
Retention time	1.8 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 1: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (ng/mL) (n = x)	Mean recovery (%)	RSD (%)	Comments
Receptor fluid and receptor wash (5% (w/v) D-glucose in water)	Phosphonic acid	2.0 (n = 5)	103	10	-
		1000 (n = 5)	98	2.5	-
		5000000000 (n = 5)	103	6.1	
Tape strips (5 strips in 5 mL 50% (v/v) aqueous MeOH)	Phosphonic acid	2.0 (n = 5)	98	4.3	-
		1000 (n = 5)	97	1.1	-
		5000000000 (n = 5)	107	5.8	-
Skin (stripped skin, pulverized and extracted with 5 mL 50% (v/v) aqueous MeOH)	Phosphonic acid	3.0 (n = 5)	85	17	-
		1000 (n = 5)	104	6.2	-
		5000000000 (n = 5)	95	6.0	-
Skin wash (100 µL soap solution, 10 mL 2% (v/v) commercial soap solution in MQ, 4 swabs (in half))	Phosphonic acid	800 (n = 5)	96	2.3	-
		2400 (n = 5)	99	5.8	-
		5000000000 (n = 5)	111	5.3	-
Donor compartment, formulation and rest tips (Water)	Phosphonic acid	2.0 (n = 5)	104	7.6	-
		1000 (n = 5)	94	2.9	-
		5000000000 (n = 4)	91	6.8	-

Table A 2: Characteristics for the method used for validation of Phosphonic acid residues in receptor fluid and receptor wash, tape strips, skin, skin wash, donor compartment, formulation and rest tips

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 8

	Phosphonic acid
Calibration range	Accepted calibration range in concentration units: receptor fluid and receptor wash: 0.590 – 1200 ng/mL tape strips: 0.881 – 1200 ng/mL skin: 0.881 – 1200 ng/mL skin wash: 199 – 3000 ng/mL donor compartment, formulation and rest tips: 0.881 – 1200 ng/mL
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	receptor fluid and receptor wash: 2.0 ng/mL tape strips: 3.0 ng/mL skin: 3.0 ng/mL skin wash: 800 ng/mL donor compartment, formulation and rest tips: 2.0 ng/mL

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of phosphonic acid in receptor fluid and receptor wash, tape strips, skin, skin wash, donor compartment, formulation and rest tips.

Methods for RH-141455

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The bioanalytical method was validated for determination of RH-141455 content in the Sprague Dawley rat plasma by liquid chromatography tandem mass spectrometry (LC-MS/MS). The validation was conducted in the calibration range of 0.100 to 49.949 µg/mL having mass transitions (m/z) 233.0/188.8 (RH-141455) and 269.3/170.2 (Tolbutamide). The LOQ of the method was 0.104 µg/mL (= lower limit of quantification (LOQQC)).*

Reference:	KCP 5.1.2 (c)/02
Report	XXXX., 2020: RH-141455: 90-day oral dietary toxicity study with toxicokinetics and 28-day recovery period in Sprague Dawley rats XXXX XXXX, Report No. U-19102, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (c)/03
Report	XXXX., 2019: RH-141455: 2 days oral dietary pharmacokinetic study in Sprague Dawley rats XXXX XXXX Report No. U-19044, No GLP, Not published
Guideline(s):	None (investigative study)
Deviations:	No
GLP:	No
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (c)/04
Report	XXXX., 2020: RH-141455: 14-day oral dietary dose range finding study in Sprague Dawley rats XXXX XXXX, Report No. U-19071, No GLP, Not published
Guideline(s):	None (investigative study)
Deviations:	The blood collection for toxicokinetics on day 2 (6 P.M) & day 3 (6 A.M) and on day 14 (6 A.M) was delayed between 1- 18 minutes from the scheduled time (± 10 minutes) across the groups. However, this deviation is regarded to have no impact on the integrity of the study.
GLP:	No
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-141455 in rat blood plasma.

RH-141455 and an internal standard (tolbutamide) were extracted from Sprague Dawley rat blood plasma using protein precipitation as extraction technique. The extracted plasma samples were analysed by LC-MS/MS. The concentration of samples was calculated by constructing a calibration curve plotting peak area ratio of RH-141455 and internal standard versus concentration by applying best fit linear regression equation with $1/X^2$ weighting factor.

Deep frozen plasma samples were allowed to thaw. A completely thawed sample was vortexed at 2000 rpm for 5 minutes. Aliquots of 25µL were pipetted in pre-labelled polypropylene tubes. 25 µL internal standard working solution (tolbutamide diluted in acetonitrile) was added to all tubes except the standard blank sample (only 25 µL acetonitrile). In each tube 500 µL of precipitation solvent was added (mobile phase B, 0.1% formic acid in acetonitrile) and vortexed at 2000 rpm for 5 minutes. Centrifugation was carried out at 4000 rpm for 5 minutes. Samples were transferred into pre-labelled auto-sampler vials and loaded onto LC-MS/MS for analysis.

Equipment (HPLC system) for RH-141455

Instrument: Shimadzu Nexera
Column: X-Bridge C18 4.6*100, 5 µm
Column temp.: 45 °C
Mobile phase: A: Milli Q water
B: 0.1% (v/v) formic acid in acetonitrile

Time [min]	% A	% B
0.01	30	70
3.0	System controller stop	

Flow rate: 0.900 mL/min
Injection volume: 10 µL
Run time: 3 minutes
Retention time: approx. 1.4 minutes (RH-141455 and tolbutamide)

LC-MS/MS Parameters

Instrument	API 4000 Triple Quadrupole
Source	Turbo Ion Spray
CAD Gas	7 L / Minute
Curtain Gas	20 L / Minute
Ion Source Gas (GS1)	45 L / Minute
Heater Gas (GS2)	50 L / Minute
Ion Spray Voltage	-4500 Volt
Temperature	450°C
Interface Heater	On

Compound Dependent Parameters	**RH-141455**	**Tolbutamide**
Ionization Mode	Negative	Negative
Q1 Mass (amu)	233.0	269.3
Q3 Mass (amu)	188.8	170.2
De-clustering Potential (Volts)	-55	-80
Entry Potential (Volts)	-10	-10
Collision Energy (Volts)	-13	-30
Collision Cell Exit Potential (CXP)	-11	-10
Dwell Time (milli seconds)	200	200

Results and discussions

Table A 3: Summary of recovery experiments (and extraction efficiency)

Level	Recovery samples (extracted)					Post extracted (comparison samples)			
	Peak area	Mean peak area	SD	RSD (%)	Mean recovery (%)	Peak area	Mean peak area	SD	RSD (%)
LQC (nom. 0.3 µg/mL)	8546	8415	438	5.20	59.23	11190	14207	1631	11.48
	7815					15365			
	8775					13643			
	7952					14530			
	8509					15597			
	8894					14915			
MQC (nom. 20 µg/mL	539985	543887	31618	5.81	63.36	780514	858342	51061	5.95
	491561					834662			
	530829					906313			
	557359					846308			
	558842					860943			
	584748					921313			
HQC (nom. 40 µg/mL)	1059289	1088584	31490	2.89	62.89	1609861	1730871	102287	5.91
	1085206					1647445			
	1130324					1695589			
	1121303					1727869			
	1081878					1851625			
	1053502					1852834			
ISTD (internal standard)	845670	843457	13389	1.59	85.13	957231	9904798	24330	2.46
	867571					984090			
	843944					1011248			
	838727					969401			
	827750					1016993			
	837080					1005825			
Overall mean recovery of RH-141455 (%)									61.83
RSD (%)									3.66

Table A 4: Summary of intra run precision and accuracy for RH-141455

Parameter	Results			
Intra Run Precision & Accuracy	**Analyte**	**QC Level**	**RSD (%)**	**RE % ***
(%Bias, ±20 %, % RSD ≤20% at LOQQC level) (Bias, ±15%, % RSD ≤15% at remaining QC)	RH-141455	LOQQC	6.03 to 8.04	-1.92 to 11.54
		LQC	2.95 to 6.87	-1.69 to 3.04
		MQC	5.84 to 7.14	-0.26 to 1.84
		HQC	2.57 to 3.69	0.93 to 6.23
(%Bias, ±20 %, % RSD ≤20% at LOQQC level) (Bias, ±15%, % RSD ≤15% at remaining QC)	RH-141455	LOQQC	9.09	5.77
		LQC	5.39	-0.34
		MQC	6.04	0.79
		HQC	3.74	3.86

* intra run/ intra-day accuracy as %bias (RE %)

Accuracy and precision / repeatability

Recovery of RH-141455 in rat blood plasma was evaluated at LQC (low quality control), MQC (mid quality control) and HQC (high quality control) levels. The % recovery of RH-141455 was consistent at all three levels. The overall recovery of RH-141455 was 61.83% with a RSD of 3.66%. The internal standard recovery was calculated at MQC level and was found 85.13 % with a RSD of 1.59%. The recovery of the internal standard was therefore within the required range of 70-110%. The accuracy of RH-141455 was determined in six replicates during the validation on two different days.

The results for accuracy and precision showed RSD values < 10%. The accuracy and precision were measured by % bias, which was determined by comparing the mean values of the measured concentrations with the nominal concentrations. The accuracy of RH-141455 was determined at LOQQC (lower limit of quantification), LQC (low quality control), MQC (mid quality control) and HQC (high quality control) level in six replicates during the validation on two different days.

The intra run/ intra-day accuracy as %bias (RE %) at QC levels varied from -1.92% to 11.54% at LOQQC, -1.69% to 3.04% at LQC, -0.26% to 1.84% at MQC and 0.93% to 6.23% at HQC level, respectively.

The inter-run/ inter-day accuracy for LOQQC, LQC, MQC and HQC were 5.77%, - 0.34%, 0.79%, and 3.86% respectively for RH-141455.

Linearity

The weighting factor was calculated for three different precision and accuracy batches. The best fit was weighted as linear regression with $1/x^2$ weighting factor. The linearity of the method was determined by a weighted ($1/x^2$) least square regression analysis of standard plots associated with a 10-point calibration curve for RH-141455. The calibration line was linear for the standards ranging from 0.100 µg/mL to 49.949 µg/mL for RH-141455, which were injected in the beginning analytical run (r^2 >0.98). These calibration curves were used to determine the concentration of validation samples.

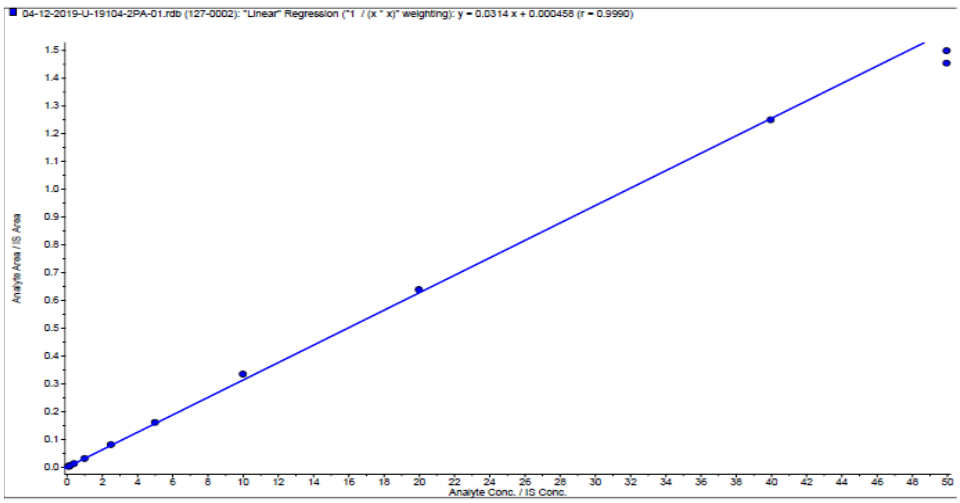


Figure A 1: Typical calibration curve for RH-141455

Limit of quantification

The LOQ of the method was defined at 0.104 µg/mL (= lower limit of quantification (LOQQC)).

Matrix effects

Matrix effects for RH-141455 and tolbutamide were calculated, both for the internal standard and RH-141455.

Specificity

The LC-MS/MS method was regarded specific for the analytes. No significant interference was observed at the retention time of RH-141455 and tolbutamide in blank and blank with internal standard samples (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

Storage stabilities were tested.
RH-141455 was found stable up to 99 days at -70 ±10°C and 94 days at -20 ±5°C in plasma.
RH-141455 was found to be stable in blood plasma at room temperature for at minimum 6.65 hours.
The stability of RH-141455 in plasma samples during freeze-thaw cycles (3 cycles) was confirmed.
Auto-sampler stability (10°C) of RH-141455 in plasma samples was found to be at least for 51.08 hours.
Stock solutions and working solutions of RH-141455 and tolbutamide were stable for at least 6.38 hours at room temperature.

Table A 5: Characteristics for the analytical method validation for the determination of RH-141455 in rat blood plasma

	RH-141455
Specificity	LC-MS/MS method is regarded specific. Typical chromatograms provided. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 10-point calibration, linear with 1/x weighting. Correlation coefficient r^2 was > 0.98 Individual calibration data presented in the study report. y = 0.0475 x + 0.000812, r = 0.9965
Calibration range	0.100 µg/mL to 49.949 µg/mL
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ = 0.104 µg/mL

Typical chromatograms are presented in the report.

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-141455 in rat blood plasma.
RH-141455 was found stable up to 99 days at -70°C ± 10°C and 94 days at -20°C ± 5°C in plasma.

(XXXX. 2019, 2020 a,b)

Method for RH-150721

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of*

	zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021: The method is fit for determination of the concentrations of RH-150721 and metabolite (RH-141455) in K_2EDTA Sprague Dawley rat plasma samples.

Reference:	KCP 5.1.2 (c)/05
Report	XXXX, 2020: RH-150721: 2-day oral dietary pharmacokinetic study in Sprague Dawley rats XXXX XXXX, Report No. U-19134, No GLP, Not published
Guideline(s):	No. (Laboratory internal SOP)
Deviations:	The draft report was provided later to the sponsor than intended by the study plan. However, this deviation does not have an impact on the integrity of the study.
GLP:	No
Acceptability:	Yes

Materials and methods

The objective of this part of the study was to determine the concentrations of RH-150721 and metabolite (RH-141455) in K_2EDTA Sprague Dawley rat plasma samples. They were analysed as per study plan using a fit for purpose LC-MS/MS method as a non GLP activity. The method for the determination of RH-150721 and RH-141455 via LC-MS/MS has been validated according to laboratory SOP using an internal standard (tolbutamide). As a result, neither RH-150721 nor RH-141455 (as one possible transformation product of RH-150721) could be observed (< LOD) in the blood plasma samples of the pharmacokinetic rat study.

K_2EDTA rat blood plasma was separated by centrifuging the whole blood sample. After extraction and centrifugation, the supernatant was transferred into auto sampler vials and volumes of 10 µL were injected on a HPLC column with LC-MS/MS detection. The samples were analysed using a calibration curve range from 0.064 µg/mL to 50.370 µg/mL (matrixed matched standards).

Equipment (HPLC system) for RH-150721

Instrument:	Shimadzu Nexera
Column:	X-Bridge 4.6*100, 5 µm
Column temp.:	45 °C
Mobile phase:	A: Type-I water B: 0.1% formic acid in acetonitrile, v/v

Time [min]	% A	% B
0.01	25	75
3.0	25	75

Flow rate:	0.800 mL/min
Injection volume:	5 µL
Run time:	3 minutes
Retention time:	approx. 1.0 min

For API 4000 Triple Quadrupole conditions, please refer to the study report.

Compound Dependent Parameters	RH-150721	Tolbutamide
Ionization Mode	Positive	Positive

Q1 Mass (amu)	381.1	271.3
Q3 Mass (amu)	187.0	91.1
De-clustering Potential (Volts)	80	50
Entry Potential	10	10
Collision Energy	30	42
Collision Cell Exit Potential (CXP)	20	10
Dwell Time	200	200

Equipment (HPLC system) for RH-141455

Instrument:	Shimadzu Nexera
Column:	X-Bridge C18 4.6*100, 5 µm
Column temp.:	45 °C
Mobile phase:	A: 0.1% ammonium acetate in Milli Q water B: acetonitrile

Time [min]	% A	% B
0.01	30	70
3.0	30	70

Flow rate:	0.900 mL/min
Injection volume:	10 µL
Run time:	4 minutes
Retention time:	approx. 1.4 minutes

For API 4000 Triple Quadrupole conditions, please refer to the study report.

Compound Dependent Parameters	RH-141455	Tolbutamide
Ionization Mode	Negative	Negative
Q1 Mass (amu)	233.0	269.3
Q3 Mass (amu)	188.8	170.2
De-clustering Potential (Volts)	-55	-80
Entry Potential	-10	-10
Collision Energy	-13	-30
Collision Cell Exit Potential (CXP)	-11	-10
Dwell Time	200	200

The following acceptance criteria of the SOP were followed:

- System suitability: Area ratio of analyte and internal standard ≤ 5 %, Analyte and ISTD RT variation ≤ 5 %.
- In Standard Blank/Standard Zero, response of the interfering peaks, if any, at the retention time of analyte or internal standard should not be more than 20 % of the extracted LLOQ response (CC1) for analyte and 5 % of the mean peak response of the ISTD observed for the passing nonzero CC standards and QC samples.
- r^2 should be > 0.98.
- 20 % deviation of the LLOQ (STD 1) from nominal concentration.
- 15% deviation of the other calibration standards other than LLOQ from nominal concentration.
- Mean % nominal concentration at each quality control sample level must be between 85% and 115% and the precision should be +15% of the %CV.
- Accuracy of at least 67% of total quality control samples and at least 50% quality control samples at

each level should meet the above acceptance criteria.

Conclusion

The method calibration data met the SOP acceptance criteria.

(XXXX. 2020)

Method for RH-150721

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The validation parameters of analytical method for the determination of RH-150721 in rat feed by reverse phase high performance liquid chromatography (HPLC) are acceptable. The requirements of SANTE/2020/12830, Rev.1 are met.*

Reference:	KCP 5.1.2 (c)/06
Report	Nagarajan, S., 2020: Analytical method validation for the estimation of RH-150721 in rat feed by reverse phase high performance liquid chromatography Gowan Crop Protection Ltd., UK Syngene International Ltd.., India, Report No. U-19162, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-150721 in rat feed by reverse phase high performance liquid chromatography (HPLC) with UV detection (200 nm) in order to check the homogeneity and stability of the test item in a rat feed preparation. As a result, the analyte was shown to be homogenic and stable in the rat feed for at minimum 2 days for 670 ppm, 4 days for 5000 ppm and 8 days for 16000 ppm RH-150721 in the rat feed (recoveries > 80%).

The required amount of the formulated feed was weighed in a 50 mL centrifuge tube. Methanol was added and the sample was vortexed for 5 minutes. The vortexed content was centrifuged for 5 minutes at 5000 rpm. A volume of 2.0 mL supernatant from the centrifuged samples was transferred into a 10 mL volumetric flask and the volume made up to the mark with diluent (for mid dose). A volume of 1.0 mL supernatant from the centrifuged samples was transferred into 10 mL volumetric flask and the volume made up to the mark with diluent (for high dose).

Equipment (HPLC System)

Instrument:	Agilent/ 1200 Series
Column:	Agilent, Zorbax SB-C18 5µm, 4.6 x 150 mm

Mobile phase: A: 0.1% ammonium acetate in Milli Q water
B: acetonitrile

Time [min]	% A	% B
0	85	15
5	55	45
15	5	95
25	85	15

Flow rate: 1.0 mL/min
Column temp.: 30 °C
Injection volume: 25 µL
Run time: 25 minutes
Retention time: 13.02 Min.

Results and discussions

Table A 6: Summary of recovery experiments

Dose level	Replicate	Concentration (mg/mL)	Recovery (%)	Mean recovery (%)	SD	RSD (%)
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose	1	0.100	99.01	98.42	0.54	0.55
	2	0.100	99.01			
	3	0.099	98.02			
	4	0.099	98.02			
	5	0.099	98.02			
High dose	1	0.985	97.91	97.63	0.49	0.50
	2	0.975	97.01			
	3	0.983	97.91			
	4	0.977	97.21			
	5	0.988	98.11			
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose (extended for stability)	1	0.104	102.97	101.39	1.13	1.11
	2	0.101	100.00			
	3	0.103	101.98			
	4	0.102	100.99			
	5	0.102	100.99			
Mid dose	1	0.517	102.58	102.94	0.58	0.56
	2	0.522	103.57			
	3	0.516	102.38			
	4	0.522	103.57			
	5	0.517	102.58			
Control feed	1	0.000	0.00	0.00	0.00	0.00
Low dose extended	1	0.124	109.73	108.49	1.01	0.93
	2	0.123	108.85			
	3	0.122	107.96			

	4	0.123	108.85			
	5	0.121	107.08			

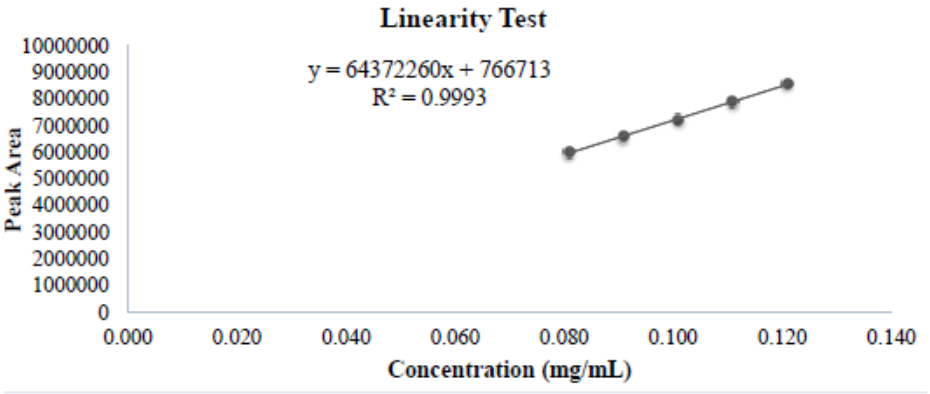
Accuracy and precision / repeatability

The results for accuracy and precision showed RSD values of < 10% for each spiking level and overall and recovery values within the required range of 70 - 110%. Three untreated samples were analysed with recovery values < LOD.

Linearity

Linearity was determined by 5-points calibration curve (single injections) using matrix matched standards at a range of 0.081 – 0.121 mg/mL ($R^2 > 0.99$). The standard solutions were injected in single replications for each level.

ID	Concentration (mg/mL)	Peak Area
Linearity level-1	0.081	6009371
Linearity level-2	0.091	6612086
Linearity level-3	0.101	7235737
Linearity level-4	0.111	7900666
Linearity level-5	0.121	8583694



Limit of quantification

The LOQ was defined as the lowest concentration tested at which an acceptable mean recovery (70-110%) with an acceptable RSD (< 20%) was obtained. Based on the available data, the LOQ was set at 0.091 mg/mL.

Limit of detection

Based on the available data, the LOD was estimated to be 0.081 mg/mL.

Matrix effects

Matrix-matched standard solutions were used.

Specificity

The method was regarded specific with a stable retention time of the analyte of 13.02 min. No interference of the matrix was observed at the retention time of analyte (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

No information given. However, high recoveries demonstrated the storage stability of the samples and sample extracts.

Table A 7: Characteristics for the analytical method validation for the determination of RH-150721 in rat feed

	RH-150721
Specificity	Typical chromatograms provided. Stable retention time of the analyte of 13.02 min. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 5-point calibration, linear. Correlation coefficient r^2 was > 0.99 Individual calibration data and calibration line equation presented in the study report. y = 64372260 x + 766713, r = 0.9993
Calibration range	0.081 mg/mL – 0.121 mg/mL
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ = 0.091 mg/mL LOD = 0.081 mg/mL

The following figure shows a typical chromatogram.

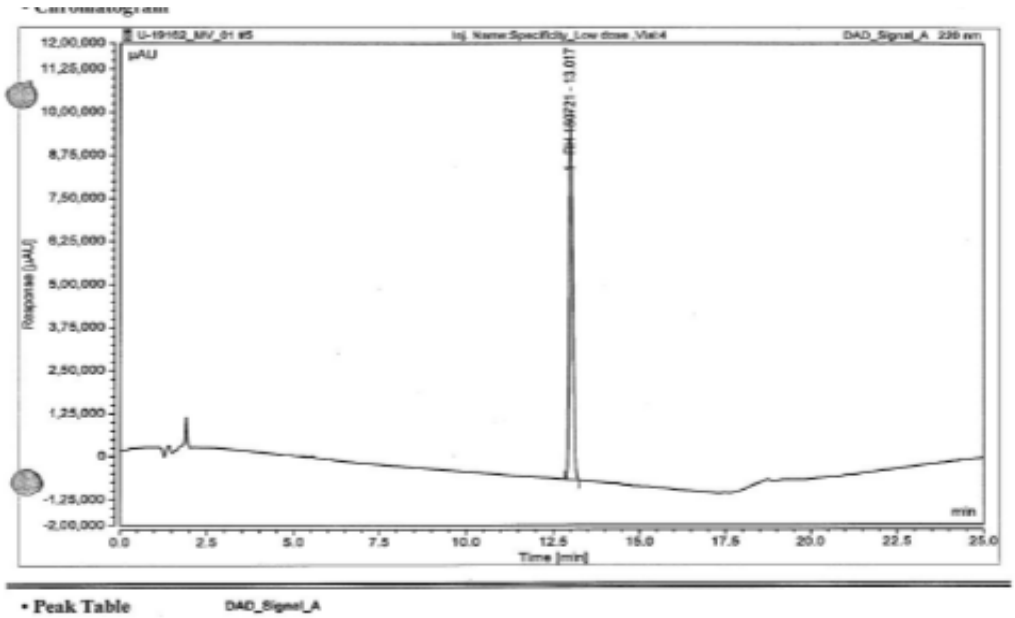


Figure A 2: **Typical Chromatogram of RH-150721**

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-150721 in rat feed.

(Nagarajan S. 2020)

Method for RH-141455

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The analytical method for the determination of RH-141455 in rat feed by reverse phase high performance liquid chromatography (HPLC) was acceptable validated. The method is suitable for the determination of RH-141455 in rat feed as the following criteria are fulfilled:* *- blank values do not exceed 30% of the lowest validated concentration.* *- mean recoveries for each level are in the range 70 – 120%.* *- the precision ≤20% RSD.*

Reference:	KCP 5.1.2 (c)/07
Report	Nagarajan, S., 2019: RH-141455: Analytical method validation for the estimation of RH-141455 in rat feed by reverse phase high performance liquid chromatography was used for the dose formulation analysis Gowan Crop Protection Ltd., UK Syngene International Ltd., India, Report No. U-19069, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study was conducted to validate an analytical method for the determination of RH-141455 in rat feed by reverse phase high performance liquid chromatography (HPLC) with UV detection (200 nm) in order to check the homogeneity and stability of the test item in a rat feed preparation. As a result, the analyte was shown to be homogenic and stable in the rat feed for at minimum 8 days with recoveries of 92.22-115.35% of nominal.

The required amount of the formulated feed was weighed in a 50 mL centrifuge tube, extraction solution was added and the sample was vortexed for 5 minutes. The vortexed content was centrifuged for 10000 rpm at 5 minutes. A volume of 1.0 mL supernatant from the centrifuged samples was transferred into a 10 mL volumetric flask and the volume made up to the mark with diluent (for low dose). A volume of 0.1 mL supernatant from the centrifuged samples was transferred into 10 mL volumetric flask and the volume made up to the mark with diluent (for high dose). Control feed was diluted as per low dose preparation. The dissolved content was centrifuged for 5 minutes at 10000 rpm.

Equipment (HPLC System)

Instrument:	Agilent/ 1200 Series
Detector:	DAD
Column:	Agilent, Zorbax SB-C18 5 µm, 4.6 x 150 mm
Mobile phase:	Isocratic mode: A: 0.1% trifluoroacetic acid in water (60%)

B: acetonitrile (40%)

Flow rate:	0.6 mL/min
Column temp.:	35 °C
Wavelength:	220 nm
Injection volume:	10 µL
Run time:	10 minutes
Retention time:	~3.34 min.

Results and discussions

Table A 8: Summary of recovery experiments

Dose Level	Replicate	Concentration (mg/mL)	Recovery (%)	Mean recovery (%)	SD	RSD (%)
Control Feed	1	0.000	0.00	0.00	0.00	0.00
Low Dose	1	0.116	112.62	112..62	0.00	0.00
	2	0.116	112.62			
	3	0.116	112.62			
	4	0.116	112.62			
	5	0.116	112.62			
High Dose	1	1.074	107.40	106.94	0.38	0.36
	2	1.072	107.09			
	3	1.067	106.59			
	4	1.064	106.51			
	5	1.071	107.10			
Control Feed	1	0.000	0.00	0.00	0.00	0.00
High Dose 1	1	1.136	113.37	113.24	0.45	0.40
	2	1.142	113.97			
	3	1.133	112.96			
	4	1.134	112.95			
	5	1.134	112.95			

Accuracy and precision / repeatability

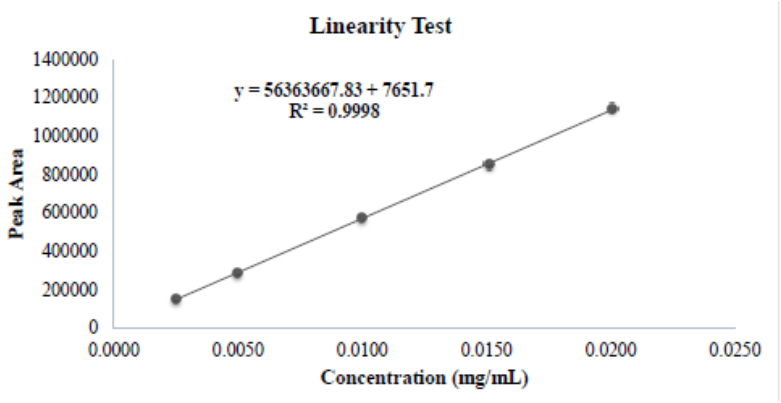
The results for accuracy and precision showed RSD values of < 10% for each spiking level and overall and recovery values within the required range of 70—110%. Two untreated samples were analysed with recovery values < LOD.

Linearity

Linearity was determined by 5-points calibration curve (single injections) using matrix matched standards at a range of 0.0025 – 0.0201 mg/mL ($r^2 > 0.99$). The standard solutions were injected in single replications for each level.

ID	Concentration (mg/mL)	Peak Area
Linearity level-1	0.0025	150367
Linearity level-2	0.0050	288144

Linearity level-3	0.0100	574553
Linearity level-4	0.0151	849796
Linearity level-5	0.0201	1145764



Limit of quantification

The LOQ was defined as the lowest concentration tested, at which an acceptable mean recovery (70-110%) with an acceptable RSD (<20%) was obtained.

Based on the available data, the LOQ was estimated to be 0.005 mg/mL.

Limit of detection

Based on the available data, the LOD was estimated to be 0.0025 mg/mL.

Matrix effects

Matrix-matched standard solutions were used.

Specificity

The method was regarded specific with a stable retention time of the analyte of 3.34 min. No interference of the matrix was observed at the retention time of analyte (i.e. < 30% of the analyte peak area).

Storage stability of samples and sample extracts

No information given. However, high recoveries demonstrated the storage stability of the samples and sample extracts.

Table A 9: Characteristics of the analytical method validation for the determination of RH-141455 in rat feed

	RH-141455
Specificity	HPLC-UV is regarded as specific and applicable. Typical chromatograms provided. Stable retention time of the analyte of 3.34 min. Blank value < 30% of the analyte peak area.
Calibration (type, number of data points)	Matrix matched standard calibration. 5-point calibration, linear. Correlation coefficient r^2 was >0.99 Individual calibration data and calibration line equation presented in the study report y = 56363667.83 + 7651.7, r = 0.9998

	RH-141455
Specificity	HPLC-UV is regarded as specific and applicable. Typical chromatograms provided. Stable retention time of the analyte of 3.34 min. Blank value < 30% of the analyte peak area.
Calibration range	0.0025 mg/mL – 0.0201 mg/mL
Assessment of matrix effects is presented	Yes.
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ estimated to be 0.005 mg/mL LOD was estimated to be 0.0025 mg/mL

The following figure shows a typical chromatogram.

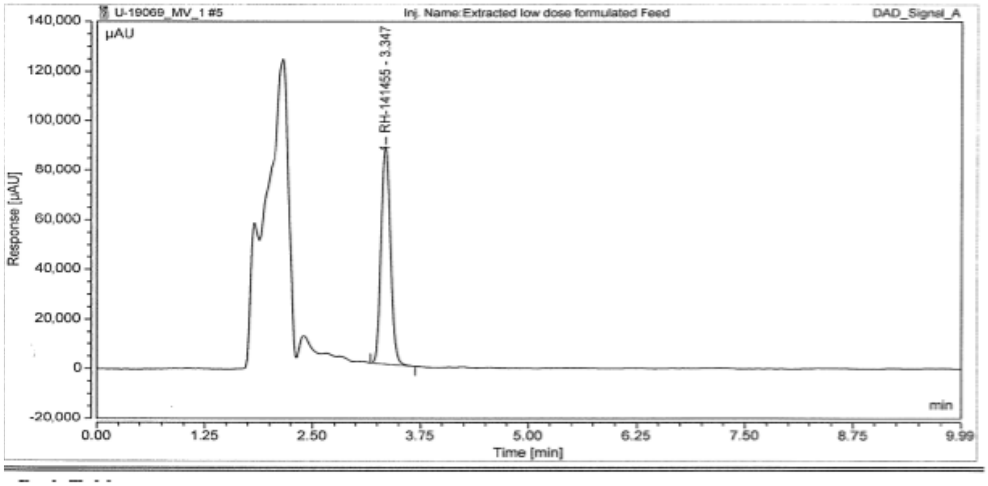


Figure A 3: **Typical Chromatogram of RH-141455**

Conclusion

The analytical method has been sufficiently validated according to SANCO/3029/99 rev. 4 for the determination of RH-141455 in rat feed.

(Nagarajan S. 2019)

Methods used in residue studies

Determination of Phosphonic acid in grape

Comments of zRMS:	The validation has been accepted. The LC-MS/MS method was applied. 2 mass transitions were monitored. The LOQ was set at 1 mg/kg for phosphonic acid (equal to 1.3 mg/kg expressed as fosetyl (ethyl-phosphonic acid). It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/01
Report:	ANALYTICAL METHOD VALIDATION TO QUANTIFY PHOSPHONIC ACID RESIDUES IN GRAPE BUNCHES (HIGH ACID CONTENT MATRIX) AND WINE, Longhi, D., 2021, report No. GLP-STUDY-20-38, Doc. No. 432-010
Guideline(s):	SANTE 2017/10632 Rev. 3 (2017), SANCO/3029/99 rev. 4 (2000), SANCO/825/00 rev.8.1 (2010), SANTE/2020/12830, rev.1 (2021)

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Grape samples were extracted with water/methanol 50/50 v/v containing 0.1% of formic acid and filtered. Wine samples were diluted with water/methanol 50/50 v/v containing 0.1% of formic acid and filtered. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Thermo Scientific Acclaim Trinity Q1 3 µm, 2.1 x 100 mm
Mobile phase (gradient)	Mobile phase A: water + 50 mM ammonium formate Mobile phase B: LC-MS grade acetonitrile
Monitored ions	81 > 79 and 81 > 63
Retention time	3.6 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 10: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Grape	Phosphonic acid 81 > 79	1 (n = 5)	98.9	2.44	-
		10 (n = 5)	99.5	0.67	-
		Overall (n= 10)	99.2	1.71	-
Grape	Phosphonic acid 81 > 63	1 (n = 5)	98.3	1.69	-
		10 (n = 5)	100.7	0.55	-
		Overall (n= 10)	99.5	1.73	-
Wine	Phosphonic acid 81 > 79	1 (n = 5)	99.5	2.12	-
		10 (n = 5)	100.4	0.70	-
		Overall (n= 10)	100.0	1.56	-
Wine	Phosphonic acid 81 > 63	1 (n = 5)	99.8	0.62	-
		10 (n = 5)	100.9	0.70	-
		Overall (n= 10)	100.3	0.85	-

Table A 11: Characteristics for the analytical method used for validation of Phosphonic acid residues in grape and wine

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 6
Calibration range	Accepted calibration range in concentration units: 32.52 – 1734 µg/L Corresponding calibration range in mass ratio units for the sample: 0.325 – 17.3 mg/kg Phosphonic acid
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	1 mg/kg

Extraction efficiency

Samples of grapes containing incurred residues were extracted using the solvent systems from the analytical method (water/methanol 50/50 v/v containing 0.1% of formic acid) and the solvent from the metabolism method (acetonitrile/HCl 0.1 N; used in metabolism study by Laurent, M.; Chabassol, Y., 1982, Report No. R000790). Analysis was carried out as described above by HPLC-MS/MS analysis.

The difference between extracted residues from the metabolism method and analytical method were less than 30%. Thus, extraction efficiency is demonstrated according to SANTE/2017/10632 rev. 4. Please refer to the following table.

Table A 12: Summary of extraction efficiency testing in grape

Method	**Grape sample code**	**Phosphonic acid residues found [mg/kg]**	**Extraction efficiency**
Residue analytical method (water/methanol 50/50 v/v containing 0.1% of formic acid)	GLP-SMPL-20-668 (untreated)	< LOD	106% (+6% compared to metabolism method)
	GLP-SMPL-20-669 (treated)	10.08	
Metabolism method (acetonitrile/HCl 0.1 N)	GLP-SMPL-20-668 (untreated)	< LOD	
	GLP-SMPL-20-669 (treated)	9.51	

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Phosphonic acid in grape and wine.

Determination of Phosphonic acid in grape

Comments of zRMS: The validation has been accepted.
The aspect of the purpose of the study was the determination of the stability of phosphonic acid in the sample extracts. This study was the validation study of the LC-MS/MS method using the isotope-labelled internal standard (ILIS) to determine phosphonic acid in grape samples. The applicant below shows the stability results.
The validation results are as follows:

Parameter	Result				
Matrix effect	- 4.9% / not significant				
Calibration (matrix-matched)	Range: 1.018 -101.8 µg/L in solution Range: 0.002036 - 0.2036 mg/kg on sample (from 20% of LOQ to 10xLOQ)				
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.				
Recovery and precision (repeatability)	Level	Concentration	Transition	% Recovery	% RSD
	LOQ (n = 5)	0.01 mg/kg	Primary (81/79)	101.4	10.9
			Confirmatory (81/63)	103.5	5.7
	10xLOQ (n = 5)	0.1 mg/kg	Primary (81/79)	103.9	2.8
			Confirmatory (81/63)	102.8	2.3
	Overall (n = 10)	/	Primary (81/79)	102.7	7.53
			Confirmatory (81/63)	103.2	4.12
	n = number of replicates				
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline				
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ) signal/noise ratio > 3				
Selectivity and specificity	Verified: no interferences found untreated samples in amounts higher than the 30% of the LOQ (< LOD)				
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory MS/MS transition. Calibration data, recovery and precision in compliance with the requirements				
Stability of the analyte in the sample extract	102.4% after 3 days in the dark at 5 ± 3°C				

It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/02
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN GRAPES, Sala, A., 2022, report No. GLP-STUDY-21-103, Doc. No. 432-008
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Grape samples were extracted with methanol containing 1% v/v of formic acid, after the addition of an isotopically labelled internal standard and water. The sample was centrifuged and filtered. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Thermo Scientific HypercarbTM 5 µm, 100 x 4.6 mm
Mobile phase (isocratic)	Mobile phase A: LC-MS grade water + 1% v/v formic acid Mobile phase B: LC-MS grade methanol + 1% v/v formic acid Ratio A/B = 70/30
Monitored ions	81 > 79 and 81 > 63

	87 > 85 for $^{18}O_3$-Phosphonic acid (ILIS)
Retention time	4.3 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 13: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Grape	Phosphonic acid 81 > 79	0.01 (n = 5)	101.4	10.9	-
		0.1 (n = 5)	103.9	2.8	-
		Overall (n= 10)	102.7	7.53	-
Grape	Phosphonic acid 81 > 63	0.01 (n = 5)	103.5	5.7	-
		0.1 (n = 5)	102.8	2.3	-
		Overall (n= 10)	103.2	4.12	-

Table A 14: Characteristics for the analytical method used for validation of Phosphonic acid residues in grape

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 1.018 – 101.8 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002036 – 0.2036 mg/kg Phosphonic acid
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Phosphonic acid in grape.

Determination of Phosphonic acid in apple

Comments of zRMS:	The validation has been accepted. This study was the validation study of the analytical method to determine phosphonic acid in apple RAC (high water matrix) and processed commodities (apple juice, apple pomace, apple sauce/puree, canned apples and dried apples (dry / high sugar content). The analytical determination was carried out applying a LC-MS/MS method using an isotope-labelled internal standard (ILIS). The analytical method was based on the multi-residual EURL-SRM method (QuPPe PO Method). The validation results were as required. The study was also applied in B7 in the context of the stability.

Reference:	KCP 5.1.2 (e)/03
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN APPLES RAC AND PROCESSED COMMODITIES, Longhi, D., 2021, report No. GLP-STUDY-21-55, Doc. No. 432-004
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Apple samples and processed commodities were extracted with methanol containing 1% v/v of formic acid, after the addition of an isotopically labelled internal standard and water. The sample was centrifuged and filtered. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Thermo Scientific HypercarbTM 5 µm, 100 x 4.6 mm
Mobile phase (isocratic)	Mobile phase A: LC-MS grade water + 0.5% v/v formic acid Mobile phase B: LC-MS grade methanol + 0.5% v/v formic acid Ratio A/B = 70/30
Monitored ions	81 > 79 and 81 > 63 87 > 85 for $^{18}O_3$-Phosphonic acid (ILIS)
Retention time	4 – 5 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 15: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Apple	Phosphonic acid 81 > 79	0.01 (n = 5)	105.3	2.3	-
		0.1 (n = 5)	100.3	2.5	-

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
		Overall (n= 10)	102.8	3.4	-
Apple	Phosphonic acid 81 > 63	0.01 (n = 5)	98.7	3.7	-
		0.1 (n = 5)	101.4	2.9	-
		Overall (n= 10)	100.1	3.4	-
Dried apple	Phosphonic acid 81 > 79	0.05 (n = 5)	106	1.57	-
		0.5 (n = 5)	105	2.56	-
		Overall (n= 10)	106	2.09	-
Dried apple	Phosphonic acid 81 > 63	0.05 (n = 5)	107	1.81	-
		0.5 (n = 5)	103	3.33	-
		Overall (n= 10)	105	3.16	-
Apple pomace	Phosphonic acid 81 > 79	0.05 (n = 3)	107	1.95	-
		0.5 (n = 3)	89.8	2.53	-
		Overall (n= 6)	98.6	9.93	-
Apple pomace	Phosphonic acid 81 > 63	0.05 (n = 3)	100	8.81	-
		0.5 (n = 3)	91.8	2.11	-
		Overall (n= 6)	95.7	7.42	-
Apple sauce puree	Phosphonic acid 81 > 79	0.05 (n = 3)	90.5	0.875	-
		0.5 (n = 3)	108	0.949	-
		Overall (n= 6)	99.4	9.85	-
Apple sauce puree	Phosphonic acid 81 > 63	0.05 (n = 3)	90.7	4.72	-
		0.5 (n = 3)	108	1.85	-
		Overall (n= 6)	99.3	9.90	-
Canned apple	Phosphonic acid 81 > 79	0.05 (n = 3)	96.2	6.72	-
		0.5 (n = 3)	106	2.77	-
		Overall (n= 6)	101	6.92	-
Canned apple	Phosphonic acid 81 > 63	0.05 (n = 3)	96.8	10.2	-
		0.5 (n = 3)	106	2.84	-
		Overall (n= 6)	101	8.11	-
Apple juice	Phosphonic acid 81 > 79	0.05 (n = 3)	79.7	13.3	-
		0.5 (n = 3)	96.1	1.49	-
		Overall (n= 6)	87.9	12.8	-
Apple pomace	Phosphonic acid 81 > 63	0.05 (n = 3)	80.3	13.3	-
		0.5 (n = 3)	97,4	2.15	-
		Overall (n= 6)	88.8	13.1	-

Table A 16: Characteristics for the analytical method used for validation of Phosphonic acid residues in apple and processed commodities

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: Apple: 0.96 – 96.0 µg/L Dried apple: 2.53 – 126 µg/L Apple pomace: 2.53 – 126 µg/L Apple sauce/puree: 1.01 – 101 µg/L Canned apple: 1.01 – 101 µg/L Apple juice: 1.01 – 101 µg/L Corresponding calibration range in mass ratio units for the sample: Apple: 0.00192 – 0.192 mg/kg Phosphonic acid Dried apple: 0.0127 – 0.63 mg/kg Apple pomace: 0.0127 – 0.63 mg/kg Apple sauce/puree: 0.00202 – 0.202 mg/kg Canned apple: 0.00202 – 0.202 mg/kg Apple juice: 0.00202 – 0.202 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	Apple: 0.01 mg/kg Processed commodities: 0.05 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Phosphonic acid in apple and processed commodities.

Determination of Phosphonic acid in apple

Comments of zRMS:	The validation has been accepted. The LC-MS/MS method was applied. 2 transitions were monitored. The validation parameters were as required. It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/04
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF PHOSPHONIC ACID IN HIGH WATER CONTENT AGRICULTURAL COMMODITIES (APPLE), Longhi, D., 2020, report No. BPL-STUDY-19-000111, Doc. No. 432-014
Guideline(s):	SANCO/825/00 rev.8.1 (2010), SANCO/3029/99 rev.4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Apple samples were extracted with water containing 1% v/v of formic acid. The sample was filtered and analysed by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Thermo Dionex® IonPacTM AG11, 2 x 50 mm + Dionex® IonPacTM AS11, 2 x 250 mm
Mobile phase (isocratic)	Mobile phase A: Water Mobile phase B: Water + 1 mM ammonium citrate Ratio A/B = 98/2
Monitored ions	81 > 79 and 81 > 63
Retention time	Approx. 3.5 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 17: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Apple	Phosphonic acid 81 > 79	1 (n = 5)	91.1	2.4	-
		10 (n = 5)	97.7	3.8	-
		Overall (n= 10)	94.4	4.8	-
Apple	Phosphonic acid 81 > 63	1 (n = 5)	93.2	3.2	-
		10 (n = 5)	100.3	4.1	-
		Overall (n= 10)	96.8	5.2	-

Table A 18: Characteristics for the analytical method used for validation of Phosphonic acid residues in apple

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 6
Calibration range	Accepted calibration range in concentration units: 30 – 1600 µg/L Corresponding calibration range in mass ratio units for the sample: 0.3 – 16 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	1 mg/kg

Compliance with SANTE/2020/12830 rev. 2

According to the new guideline, linearity should preferably be demonstrated by residuals. In the report summarized above, the peak areas are included. Based on the determined peak areas of the individual calibration standards and the presented calibration graph, it can be concluded that the residuals would be randomly distributed. Therefore, linearity is demonstrated. As all other validation parameters are in compliance with the new guideline, this is considered to have no adverse effect on the method validation. In addition, the minimum validation data for methods validated before the implementation of SANTE/2020/12830 rev. 2 are fulfilled in any case.

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Phosphonic acid in apple.

Determination of Phosphonic acid in potato

Comments of zRMS:	The validation has been accepted. This study was the validation study of the LC-MS/MS method to determine phosphonic acid in potato tuber and processed samples (potato waste and potato dried pulp). The method was based on the EURL-SRM QuPPe-PO-Method. The determination with LC-MS/MS was performed in the presence of an internal standard ($^{18}O_3$ -Phosphonic acid). The summary of the validation results is as follows:

Parameter	Result
Matrix effect	**Matrix** / **Matrix effect** Potato tuber: - 38% (significant) Potato waste: - 37% (significant) Potato dried pulp: - 33% (significant)
Calibration (matrix-matched)	**Matrix** / **Range (µg/L)** / **Range (mg/kg)** Potato tuber: 1.00 – 100 (20% LOQ – 100% above 10xLOQ) / 0.002 – 0.2 Potato waste: 0.5 – 50 (20% LOQ – 100% above 10xLOQ) / 0.002 – 0.2 Potato dried pulp: 0.5 – 50 (20% LOQ – 100% above 10xLOQ) / 0.002 – 0.2 The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.
Recovery and precision (repeatability)	Potato tuber Level / Concentration / Transition / % Recovery / % RSD LOQ (n = 5) / 0.01 mg/kg / Primary (81/79) / 79.5 / 15.0 Confirmatory (81/63) / 91.3 / 3.4 10xLOQ (n = 5) / 0.1 mg/kg / Primary (81/79) / 79.6 / 3.9 Confirmatory (81/63) / 78.7 / 4.2 Overall (n = 10) / / Primary (81/79) / 79.6 / 10.3 Confirmatory (81/63) / 85.0 / 8.6 Potato wastes Level / Concentration / Transition / % Recovery / % RSD LOQ (n = 5) / 0.01 mg/kg / Primary (81/79) / 99.2 / 1.6 Confirmatory (81/63) / 102 / 1.0 10xLOQ (n = 5) / 0.1 mg/kg / Primary (81/79) / 98.5 / 0.72 Confirmatory (81/63) / 100 / 1.6 Overall (n = 10) / / Primary (81/79) / 98.9 / 1.2 Confirmatory (81/63) / 101 / 1.7 Potato dried pulp Level / Concentration / Transition / % Recovery / % RSD LOQ (n = 5) / 0.01 mg/kg / Primary (81/79) / 98.2 / 3.5 Confirmatory (81/63) / 95.7 / 4.3 10xLOQ (n = 5) / 0.1 mg/kg / Primary (81/79) / 102 / 1.7 Confirmatory (81/63) / 100 / 0.85 Overall (n = 10) / / Primary (81/79) / 99.9 / 3.1 Confirmatory (81/63) / 98.0 / 3.8 n = number of replicates
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline
Limit of detection (LOD)	Verified for each matrix at 0.002 mg/kg (20% of LOQ) signal/noise ratio higher than 3
Selectivity and specificity	Verified for each matrix: no interferences found untreated samples in amounts higher than the 30% of the LOQ (< LOD)
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory SRM transition. Calibration data, recovery and precision in compliance with the requirements
Stability of the analyte in the samples extract	Verified for 3 days at 5 ± 3°C in the dark (potato tuber: 100.1%, potato waste: 101.6%, potato dried pulp: 106.6%)
Stability of the analyte in the standard solution	Verified for 74 days at 5 ± 3°C in the dark (stock solution in water): the difference from the stored and a fresh solution was 0.8%

It could be concluded that the method is applicable for the intended purpose.

Reference: KCP 5.1.2 (e)/05
Report: VALIDATION OF AN ANALYTICAL METHOD FOR THE

	DETERMINATION OF PHOSPHONIC ACID IN POTATO, Longhi, D., 2022, report No. GLP-STUDY-21-52, Doc. No. 432-015
Guideline(s):	SANTE/2020/12830, rev.1 (2021), SANTE 2017/10632 rev. 3 (2017)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples and processed commodities were extracted with methanol containing 1% v/v of formic acid, after the addition of an isotopically labelled internal standard and water. The sample was centrifuged and filtered. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions*

System	HPLC-MS/MS
Column	Thermo Scientific HypercarbTM 5 µm, 100 x 4.6 mm
Mobile phase (isocratic)	Mobile phase A: LC-MS grade water + 0.5% v/v formic acid Mobile phase B: LC-MS grade methanol + 0.5% v/v formic acid Ratio A/B = 70/30
Monitored ions	81 > 79 and 81 > 63 87 > 85 for $^{18}O_3$-Phosphonic acid (ILIS)
Retention time	Approx. 4.7 min

* Samples of potato wastes and dried pulp were analysed on a different instrument but with the same column and conditions

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 19: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Potato tuber	Phosphonic acid 81 > 79	0.01 (n = 5)	79.5	15.0	-
		0.1 (n = 5)	79.6	3.9	-
		Overall (n= 10)	79.6	10.3	-
Potato tuber	Phosphonic acid 81 > 63	0.01 (n = 5)	91.3	3.4	-
		0.1 (n = 5)	78.7	4.2	-
		Overall (n= 10)	85.0	8.6	-
Potato wastes	Phosphonic acid 81 > 79	0.01 (n = 5)	99.2	1.6	-
		0.1 (n = 5)	98.5	0.72	-
		Overall (n= 10)	98.9	1.2	-
Potato wastes	Phosphonic acid 81 > 63	0.01 (n = 5)	102	1.0	-
		0.1 (n = 5)	100	1.6	-
		Overall (n= 10)	101	1.7	-
Potato dried	Phosphonic acid	0.01 (n = 5)	98.2	3.5	-

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
pulp	81 > 79	0.1 (n = 5)	102	1.7	-
		Overall (n= 10)	99.9	3.1	-
Potato dried pulp	Phosphonic acid 81 > 63	0.01 (n = 5)	95.7	4.3	-
		0.1 (n = 5)	100	0.85	-
		Overall (n= 10)	98.0	3.8	-

Table A 20: Characteristics for the analytical method used for validation of Phosphonic acid residues in potato matrices

	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: Potato: 1 – 100 µg/L Potato wastes and potato dried pulp: 0.5 – 50 µg/L Corresponding calibration range in mass ratio units for the sample: All matrices: 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Phosphonic acid in potato tuber, potato wastes and potato dried pulp.

Determination of metabolite RH-141452 in grape

Comments of zRMS:	The validation has been accepted. The objective of this study was the validation of the analytical method to determine RH-141452 (total fraction) in grape samples. The analytical determination was carried out using a HPLC-MS/MS method, validated in compliance with SANTE/2020/12830, Rev.1 guideline. The method was based on an alkaline hydrolysis and an extraction followed by a HPLC-HRMS/MS. The extraction efficiency of the analyte in high acid content matrices has been verified according to SANTE 2017/10632 rev. 3 in the GLP study BPL-STUDY-18-000085. Results of the validation parameters and the extraction efficiency are summarised as follows:

HPLC-MS/MS determination of RH-141452 - validation results summary

Parameter	Result	SANTE/2020/12830 rev.1
Matrix effect	- 23.2 % / significant matrix effect	< ±20%
Calibration (matrix-matched)	Range: 0.536 – 53.60 µg/L in solution Range: 0.002144 – 0.2144 mg/kg on sample (from 20 % of LOQ to 20xLOQ)	At least from 30% of LOQ to at least 20% above the highest level
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	Residuals randomly distributed
Recovery and precision repeatability	See nested table below	LOQ level (0.01 mg/kg) recoveries 70 – 110% RSD ≤20% 10xLOQ level (0.1 mg/kg) recoveries 70 – 110% RSD ≤15% (limits more restrictive than the guideline requirements)
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline	LOQ: lowest validated level with sufficient recovery and precision
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ) signal/noise ratio higher than 3	LOD < 30% of LOQ
Selectivity and specificity	Verified: no interferences found in untreated samples in amounts higher than 30% of the LOQ (< LOD)	Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory MS/MS transition. Calibration data, recovery and precision in compliance with the requirements	Confirmation by monitoring at least 1 SRM transition, providing linearity, recovery, precision, selectivity
Stability of the analyte in the sample extract	100.4 % (primary ion) after 3 days in the dark at 5 ± 3°C	70-120%
Stability of the analyte in the standard solution	+ 6.8% after 130 days in the dark at 5 ± 3°C	Mean peak area difference of the stored solution and a freshly prepared solution: < 10%
Parameter	**Result**	**SANTE 2017/10632 rev. 3**
Extraction efficiency	The extraction efficiency of Zoxamide in grape samples using the extraction procedure adopted in this study was successfully demonstrated in the study coded BPL-STUDY-18-000085	Extraction efficiency considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the metabolism study differ no more than 30%

Nested table for Recovery and precision repeatability:

Level	Concentration (mg/kg)	Transition	% Recovery	% RSD
LOQ (n = 5)	0.01	Primary (218.9621)	84.9	5.5
		Confirmatory (144.9617)	86.3	9.0
10xLOQ (n = 5)	0.1	Primary (218.9621)	92.3	2.4
		Confirmatory (144.9617)	99.3	3.2
Overall (n = 10)	/	Primary (218.9621)	88.6	5.9
		Confirmatory (144.9617)	92.8	9.5

n = number of replicates

It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/06
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RH-141452 (TOTAL FRACTION) IN GRAPES, Sala, A., 2022, report No. GLP-STUDY-21-102, Doc. No. 432-007
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Grape samples were submitted to an alkaline hydrolysis step: After addition of acetonitrile and 5M NaOH, samples were heated at 40°C. After cooling to room temperature, the reaction mixture was neutralized by addition of 2.5M sulphuric acid. After addition of a Quechers extraction salt packet, the sample was centrifuged, and the organic phase was separated. The solid residue was extracted with acetonitrile and the organic phases were combined. After dilution and acidification with formic acid, analysis was carried out by HPLC-HRMS/MS.

Chromatographic conditions

System	HPLC-HRMS/MS
Column	Waters Acquity UPLC HSS PFP, 1.8 µm, 2.1 x 150 mm
Mobile phase (gradient)	Mobile phase A: 0.2% v/v of formic acid in LC-MS grade water + 5 mM ammonium formate Mobile phase B: 0.2% v/v of formic acid in methanol UPLC grade + 5 mM ammonium formate
Monitored ions	m/z 218.9621 (primary) m/z 218.9621 > 144.9617 (confirmatory)
Retention time	4.1 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 21: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Grape	RH-141452 m/z 218.9621	0.01 (n = 5)	84.9	5.5	-
		0.1 (n = 5)	92.3	2.4	-
		Overall (n= 10)	88.6	5.9	-
Grape	RH-141452 m/z 144.9617	0.01 (n = 5)	86.3	9.0	-
		0.1 (n = 5)	99.3	3.2	-
		Overall (n= 10)	92.8	9.5	-

Table A 22: Characteristics for the analytical method used for validation of RH-141452 residues in grape

	RH-141452
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.536 – 53.60 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002144 – 0.2144 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of RH-141452 in grape.

Determination of metabolite RH-141452 in apple

Comments of zRMS:	The validation has been accepted. The objective of this study was the validation of the analytical method to determine RH-141452 (total fraction) in apple. The analytical determination was carried out using a HPLC-HRMS method, validated in compliance with SANTE/2020/12830, Rev.1 guideline. The method consisted of a base hydrolysis of the sample and an extraction of the analyte from the matrix, followed by a HPLC-HRMS analysis. The extraction efficiency of the analytical method has been verified according to SANTE 2017/10632 rev. 3. Results of the validation parameters and the extraction efficiency were below summarised.

RH-141452 determination in apple (HPLC- HRMS) - validation results summary

Parameter	Result	SANTE/2020/12830 rev.1 limit
Matrix effect	- 8.26 % / not significant	< ±20%
Calibration (matrix-matched)	Range: 1.084 – 75.88 µg/L in solution Range: 0.002 – 0.2 mg/kg on sample (from 20 % of LOQ to 40 % above 10xLOQ)	At least from 30% of LOQ to at least 20% above the highest level
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	Residuals randomly distributed
Recovery and precision (repeatability)	Level / Concentration (mg/kg) / Signal / % Recovery / % RSD LOQ (n = 5) / 0.01 / Primary signal (218.9621) / 89.6 / 3.3 LOQ (n = 5) / 0.01 / Confirmatory signal (144.9617) / 89.6 / 2.0 10xLOQ (n = 5) / 0.1 / Primary signal (218.9621) / 91.4 / 2.7 10xLOQ (n = 5) / 0.1 / Confirmatory signal (144.9617) / 88.9 / 3.5 Overall (n = 10) / / Primary signal (218.9621) / 90.5 / 3.0 Overall (n = 10) / / Confirmatory signal (144.9617) / 89.3 / 2.7 n = number of replicates	LOQ level (0.01 mg/kg) recoveries 70 – 110% RSD ≤20% 10xLOQ level (0.1 mg/kg) recoveries 70 – 110% RSD ≤15% (limits more restrictive than the guideline requirements)
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline	LOQ: lowest validated level with sufficient recovery and precision
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ) signal/noise ratio higher than 3	LOD < 30% of LOQ
Selectivity and specificity	Verified: no interferences found in untreated samples in amounts higher than 30% of the LOQ (< LOD)	Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory signal. Calibration data, recovery and precision in compliance with the requirements	Confirmation by monitoring at least 1 additional fragment ion, providing linearity, recovery, precision, selectivity
Stability of the analyte in the sample extract	97.9 % after 3 days in the dark at 5 ± 3°C	70-120%
Stability of the analyte in the standard solution	+ 6.8% after 130 days in the dark at 5 ± 3°C	< 10%
Parameter	**Result**	**SANTE 2017/10632 rev. 3 limit**
Extraction efficiency	The extraction efficiency of RH-141452 from high water commodity matrices using the extraction procedure adopted in this study has already been successfully demonstrated in the mentioned study BPL-STUDY-18-000085	Extraction efficiency considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the metabolism study differ no more than 30%

It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/07
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RH-141452 (TOTAL FRACTION) IN APPLES, Longhi, D., 2021, report No. GLP-STUDY-21-54, Doc. No. 432-005
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Apple samples were submitted to an alkaline hydrolysis step: After addition of acetonitrile and 5M NaOH, samples were heated at 40°C. After cooling to room temperature, the reaction mixture was neutralized by addition of 2.5M sulphuric acid. After addition of a Quechers extraction salt packet, the sample was

centrifuged, and the organic phase was separated. The solid residue was extracted with acetonitrile and the organic phases were combined. After dilution and acidification with formic acid, analysis was carried out by HPLC-HRMS/MS.

Chromatographic conditions

System	HPLC-HRMS/MS
Column	Waters Acquity UPLC HSS PFP, 1.8 µm, 2.1 x 150 mm
Mobile phase (gradient)	Mobile phase A: 0.2% v/v of formic acid in LC-MS grade water + 5 mM ammonium formate Mobile phase B: 0.2% v/v of formic acid in methanol UPLC grade + 5 mM ammonium formate
Monitored ions	m/z 218.9621 (primary) m/z 218.9621 > 144.9617 (confirmatory)
Retention time	4.1 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 23: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Apple	RH-141452 m/z 218.9621	0.01 (n = 5)	89.6	3.3	-
		0.1 (n = 5)	91.4	2.0	-
		Overall (n= 10)	90.5	3.0	-
Apple	RH-141452 m/z 144.9617	0.01 (n = 5)	89.6	2.0	-
		0.1 (n = 5)	88.9	3.5	-
		Overall (n= 10)	89.3	2.7	-

Table A 24: Characteristics for the analytical method used for validation of RH-141452 residues in apple

	RH-141452
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 1.084 – 75.88 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of RH-141452 in apple.

Determination of Zoxamide in potato

Comments of zRMS:	The validation has been accepted. The objective of this study was to validate an analytical method for the determination of the zoxamide racemate. LC-MS/MS method was applied. 2 transitions were monitored. The validation parameters were as required. It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/08
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN POTATO, Longhi, D., 2022, Doc. No. 432-016
Guideline(s):	SANTE 2017/10632 rev. 3 (2017), SANTE/2020/12830, rev.1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were extracted twice with methanol/acetonitrile/water 40/40/20 (v/v/v) containing 0.2% formic acid and centrifuged. The combined extracts were diluted with the extraction solvent and centrifuged. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Mobile phase (gradient)	Mobile phase A: water with 0.1 % formic acid Mobile phase B: acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.4 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 25: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Potato	Zoxamide 336 > 186.9	0.01 (n = 5)	105.9	4.1	-
		0.1 (n = 5)	109.5	4.9	-
		Overall (n= 10)	107.7	4.6	-
Potato	Zoxamide	0.01 (n = 5)	106.4	6.3	-

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
	336 > 159.0	0.1 (n = 5)	109.8	4.5	-
		Overall (n= 10)	108.1	5.4	-

Table A 26: Characteristics for the analytical method used for validation of Zoxamide residues in potato

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.5375 – 53.75 µg/L Corresponding calibration range in mass ratio units for the sample: 0.00215 – 0.215 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in potato.

Determination of Zoxamide, MDI-0043 (RH-141452), MDI-0050 (RH-141455) and MDI-0074 (phosphonic acid) in potato

Comments of zRMS:	The validation has been accepted. LC-MS/MS methods were applied. 2 transitions were monitored. The validation parameters were as required. It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (e)/09
Report:	VALIDATION OF ANALYTICAL METHODS FOR DETERMINATION OF GWN-8030, MDI-0043, MDI-0050 AND MDI-0074 IN POTATO MATRICES, Link, T., 2023, report No. IF23-06197316, Doc. No. 432-017
Guideline(s):	SANTE/2020/12830, Rev.2 (2023), ENV/JM/MONO(2007)17
Deviations:	No
GLP:	Yes
Acceptability:	Yes

During this study three separate analytical methods for the determination of the analytes GWN-8030 (Zoxamide), MDI-0043 (RH-141452) and MDI-0050 (RH-141455) (one method) and MDI-0074 (phosphonic acid) in potato tuber (RAC) and processed potato commodities were validated.
Fourteen potato matrices (tuber, microwaved/boiled potato, baked potato, fried potato, French fries, starch, protein, wet peel, process waste, crisps, canned potato, ensiled potato, dried pulp and flakes) were used as test system.

Method for Zoxamide

Materials and methods

To samples of potato tuber water and acetonitrile were added and shaken vigorously. To the raw extract QuEChERS-Mix (citrate buffered) was added and shaken thoroughly. For phase separation a centrifugation was performed. Thereafter, a subsample of the upper layer of the extract was diluted with methanol/water (50/50, v/v).
Final determination is achieved by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

Chromatographic conditions

System	HPLC-MS/MS
Column	Thermo Scientific, Accucore Phenyl/Hexyl 50 x 2.1 mm, 2.6 µm
Mobile phase (gradient)	Mobile phase A: water with 0.1 % formic acid Mobile phase B: acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 187 and 336.0 > 157
Retention time	3.9 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 27: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Tuber	Zoxamide 336 > 187	0.01 (n = 5)	90.9	1.1	-
		0.1 (n = 5)	93.3	2.5	-
		Overall (n= 10)	92.1	2.3	-
Tuber	Zoxamide 336 > 157	0.01 (n = 5)	89.5	1.7	-
		0.1 (n = 5)	93.7	2.9	-
		Overall (n= 10)	91.6	3.3	-

Table A 28: Characteristics for the analytical method used for validation of Zoxamide residues in potato

	Zoxamide
Specificity	The detector signals in the control samples were below 30 % of the LOQ. Analysis of a reagent blank showed no residues (< LOD) for Zoxamide. Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched stanards used individual calibration data presented| calibration line equation presented

	Zoxamide
Specificity	The detector signals in the control samples were below 30 % of the LOQ. Analysis of a reagent blank showed no residues (< LOD) for Zoxamide. Mass spectrum is provided. blank value < 30 % LOQ
	number of data points: 7
Calibration range	concentrations ranging from 0.15 to 15 ng/mL (equivalent to 0.03 to 0.30 mg/kg) for Zoxamide
Assessment of matrix effects is presented	Yes
Stability of Fortification and Calibration Solutions	The stability of Zoxamide in standard solutions was tested for at least 69 days in methanol and methanol/water (50/50, v/v). Old working solutions were reanalysed and compared with working solutions prepared freshly before analysis. The stability was proven as the differences of the stored solutions in all used solvents to the fresh prepared solutions were ≤ 10 %.
Stability Tests with Sample Final Volume of Zoxamide	The stability of Zoxamide in final volumes and in sample extracts was tested within this study for at least 7 days. Extracts are found to be stable if the recovery range lies within 70 – 110% of the nominal concentration.
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in potato.

Method for RH-141452 and RH-141455

Materials and methods

To samples of potato tuber sodium hydroxid (5.0 mol/l NaOH) and acetonitrile were added. Hydrolysis and extraction were achieved in a shaking water bath. Afterwards the extract was neutralised with sulfuric acid (2.5 mol/L H2SO4). To the neutralised extract formic acid and QuEChERS-Mix (unbuffered) were added and shaken thoroughly. For phase separation a centrifugation was performed. Thereafter, the upper layer of the extract was evaporated to dryness and reconstituted with methanol/water (50/50, v/v). Final determination is achieved by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

Chromatographic conditions

System	HPLC-MS/MS
Column	Analytical Column: Agela Technologies, Venusil HILIC 100 x 2.1 mm, 3.0 µm
Mobile phase (gradient)	Mobile phase A: water with formic acid 1000/3 Mobile phase B: acetonitrile with formic acid 1000/3
Monitored ions	RH-141452 233 → 109 233 → 152 RH-141455 219 → 145 219 → 175
Retention time	RH-141452 approx. 0.9 min. RH-141455 approx. 4.4 min.

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 29: Recovery results from method validation of RH-141452 and RH-141455 using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Tuber	RH-141152 233 109	0.01 (n = 5)	75.9	7.7	-
		0.1 (n = 5)	71.1	5.5	-
		Overall (n= 10)	73.5	7.3	-
Tuber	RH-141152 233 152	0.01 (n = 5)	75.3	7.9	-
		0.1 (n = 5)	71.8	7.5	-
		Overall (n= 10)	73.6	7.7	-
Tuber	RH-141155 219 145	0.01 (n = 5)	88.6	4.2	-
		0.1 (n = 5)	91.6	1.8	-
		Overall (n= 10)	90.1	3.5	-
Tuber	RH-141155 219 175	0.01 (n = 5)	102	4.6	-
		0.1 (n = 5)	89.2	1.3	-
		Overall (n= 10)	95.5	7.8	-

Table A 30: Characteristics for the analytical method used for validation of RH-141452 and RH-141455 residues in potato

	RH-141452	RH-141455
Specificity	Mass spectrum is provided. blank value < 30 % LOQ	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matches standards used individual calibration data presented| calibration line equation presented number of data points: 7	Matrix matches standards used individual calibration data presented| calibration line equation presented number of data points: 7
Calibration range	0.60 to 26 ng/mL (equivalent to 0.03 to	0.60 to 26 ng/mL (equivalent to 0.03 to

	0.013 mg/kg)	0.013 mg/kg)
Assessment of matrix effects is presented	Yes	Yes
Stability of Fortification and Calibration Solutions	The stability of RH141452 in standard solutions was tested for at least 69 days in methanol and methanol/water (50/50, v/v). Old working solutions were reanalysed and compared with working solutions prepared freshly before analysis. The stability was proven as the differences of the stored solutions in all used solvents to the fresh prepared solutions were ≤ 10 %.	The stability of RH141454 in standard solutions was tested for at least 69 days in methanol and methanol/water (50/50, v/v). Old working solutions were reanalysed and compared with working solutions prepared freshly before analysis. The stability was proven as the differences of the stored solutions in all used solvents to the fresh prepared solutions were ≤ 10 %.
Stability Tests with Sample Final Volume of Zoxamide	The stability of RH141452 in final volumes and in sample extracts was tested within this study for at least 7 days. Extracts are found to be stable if the recovery range lies within 70 – 110% of the nominal concentration.	The stability of RH141455 in final volumes and in sample extracts was tested within this study for at least 7 days. Extracts are found to be stable if the recovery range lies within 70 – 110% of the nominal concentration.
Limit of determination/quantification	0.01 mg/kg	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of RH141452 and RH141455 in potato.

Method for Phosphonic Acid

Materials and methods

To subsamples of potato matrices water was added to increase the water content of the sample, if needed. Samples were extracted with methanol/formic acid (99/1, v/v) and homogenised by a mechanical shaker. Aliquots of the extracts were diluted with acetonitrile/ultra-pure water/formic acid (70/27.5/2.5, v/v/v) and internal standard solution to final volume and centrifuged before analysis.
Final determination was by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

Chromatographic conditions

System	HPLC-MS/MS
Column	Analytical Column: Thermo Scientific Hypercarb, 100 x 4.6 mm, 5 µm
Mobile phase (gradient)	Mobile phase A: water with formic acid 1000/20 Mobile phase B: acetonitrile with formic acid 1000/20
Monitored ions	87 → **79** **81** → **63**
Retention time	4.2 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 31: Recovery results from method validation of Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Tuber	Phosphonic acid 81 → 79	0.02 (n = 5)	97.4	1.9	-
		0.2 (n = 5)	101	0.7	-
		Overall (n= 10)	99.2	2.4	-
	Phosphonic acid 81 → 63	0.02 (n = 5)	101	3.4	-
		0.2 (n = 5)	97.4	1.2	-
		Overall (n= 10)	99.2	3.1	-
Microwaved Potatoes	Phosphonic acid 81 → 79	0.02 (n = 3)	103	2.5	-
		0.2 (n = 3)	102	1.1	-
		Overall (n= 6)	103	1.8	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	106	2.4	-
		0.2 (n = 3)	102	0.4	-
		Overall (n= 6)	104	2.4	-
Baked potatoes	Phosphonic acid 81 → 79	0.02 (n = 3)	106	2.1	-
		0.2 (n = 3)	101	1.2	-
		Overall (n= 6)	104	2.7	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	111	2.7	-
		0.2 (n = 3)	99.4	2.4	-
		Overall (n= 6)	105	6.2	-
Fried Potatoes	Phosphonic acid 81 → 79	0.02 (n = 3)	81.5	0.9	-
		0.2 (n = 3)	79.3	2.8	-
		Overall (n= 6)	80.4	2.4	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	83.7	1.4	-
		0.2 (n = 3)	79.2	3.4	-
		Overall (n= 6)	81.4	3.7	-
French Fries	Phosphonic acid 81 → 79	0.02 (n = 3)	81.5	0.9	-
		0.2 (n = 3)	79.3	2.8	-
		Overall (n= 6)	80.4	2.4	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	83.7	1.4	-
		0.2 (n = 3)	79.2	3.4	-
		Overall (n= 6)	81.4	3.7	-
Starch	Phosphonic acid 81 → 79	0.02 (n = 5)	95.2	7.1	-
		0.2 (n = 5)	103	3.0	-
		Overall (n= 10)	99.2	6.6	-
	Phosphonic acid 81 → 63	0.02 (n = 5)	99.0	6.4	-
		0.2 (n = 5)	103	2.6	-

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
		Overall (n= 10)	101	4.9	-
Protein	Phosphonic acid 81 → 79	0.02 (n = 3)	102	0.9	-
		0.2 (n = 3)	103	1.6	-
		Overall (n= 6)	103	1.3	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	109	1.1	-
		0.2 (n = 3)	104	1.8	-
		Overall (n= 6)	106	2.9	-
Wet peel	Phosphonic acid 81 → 79	0.02 (n = 3)	104	3.5	-
		0.2 (n = 3)	105	0.3	-
		Overall (n= 6)	105	2.3	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	106	1.0	-
		0.2 (n = 3)	107	0.8	-
		Overall (n= 6)	106	1.2	-
Process Waste	Phosphonic acid 81 → 79	0.04 (n = 3)	106	2.5	-
		0.4 (n = 3)	106	1.9	-
		Overall (n= 6)	106	2.0	-
	Phosphonic acid 81 → 63	0.04 (n = 3)	96.2	2.6	-
		0.4 (n = 3)	103	1.4	-
		Overall (n= 6)	99.8	4.4	-
Crisps	Phosphonic acid 81 → 79	0.05 (n = 5)	103	1.8	-
		0.5 (n = 5)	100	0.9	-
		Overall (n= 10)	102	1.9	-
	Phosphonic acid 81 → 63	0.05 (n = 5)	104	3.3	-
		0.5 (n = 5)	99.2	1.1	-
		Overall (n= 10)	102	3.4	-
Canned Potatoes	Phosphonic acid 81 → 79	0.05 (n = 3)	106	2.2	-
		0.5 (n = 3)	104	1.6	-
		Overall (n= 6)	105	1.9	-
	Phosphonic acid 81 → 63	0.05 (n = 3)	105	1.7	-
		0.5 (n = 3)	102	1.6	-
		Overall (n= 6)	104	1.9	-
Ensiled Potatoes	Phosphonic acid 81 → 79	0.02 (n = 3)	107	1.4	-
		0.2 (n = 3)	107	0.5	-
		Overall (n= 6)	107	1.0	-
	Phosphonic acid 81 → 63	0.02 (n = 3)	113	1.4	-
		0.2 (n = 3)	108	0.4	-

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
		Overall (n= 6)	110	2.7	-
Flakes	Phosphonic acid 81 → 79	0.06 (n = 3)	91.7	6.8	-
		0.6 (n = 3)	81.6	11	-
		Overall (n= 6)	86.7	10	-
	Phosphonic acid 81 → 63	0.06 (n = 3)	99.0	8.5	-
		0.6 (n = 3)	83.2	11	-
		Overall (n= 6)	91.1	13	-
Dried pulp	Phosphonic acid 81 → 79	0.02 (n = 3)	102	1.6	-
		0.2 (n = 3)	98.2	1.4	-
		Overall (n= 6)	100	2.1	-
	Phosphonic acid 81 → 63	0.02 (n = 5)	100	1.9	-
		0.2 (n = 5)	98.1	1.2	-
		Overall (n= 10)	99.3	1.9	-

Table A 32: Characteristics for the analytical method used for validation of phosphonic acid residues in potato

	phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	Possible matrix effect of the analyte is eliminated by using an internal standard solution of isotopically labelled reference item. Matrix effect was not addressed. individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	0.0374 to 17 ng/mL (equivalent to 30 % of LOQ to 1400 % LOQ) for phosphonic acid.
Assessment of matrix effects is presented	Possible matrix effect of the analyte is eliminated by using an internal standard solution of isotopically labelled reference item. Matrix effect was not addressed.
Stability of Fortification and Calibration Solutions	The stability of Phosphonic Acid standard solutions was tested for at least 98 days in water and for at least 92 days in acetonitrile/water/formic acid (50/48/2, v/v/v). Old working solutions were reanalysed and compared with working solutions prepared freshly before analysis. The stability was proven as the differences of the stored solutions in all used solvents to the fresh prepared solutions were ≤ 10 %.
Limit of determination/quantification	Tuber 0.02 mg/kg Crisps 0.05 mg/kg Starch 0.02 mg/kg Wet peel 0.02 mg/kg Microwaved/ Boiled Potatoes 0.02 mg/kg Baked Potatoes 0.02 mg/kg Process Waste 0.04 mg/kg

	Flakes	0.06 mg/kg
	Potato Protein	0.02 mg/kg
	Fried Potatoes	0.02 mg/kg
	Chips/ French Fries	0.02 mg/kg
	Canned potatoes	0.05 mg/kg
	Ensiled potatoes	0.02 mg/kg
	Dried pulp	0.02 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of phosphonic acid in potato tuber, crisps, starch, wet peel, microwaved/boiled potatoes, baked potatoes, process paste, flakes, potato protein, fried potatoes, chips/ french fries, canned potatoes, ensiled potatoes and dried pulp.

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

*Comments of RMS **Latvia**:*	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The analytical method based on HPLC-DAD was validated according to the SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) requirements. The limit of quantification of this method is 1 mg/L of RH-141452 in all the buffers.*

Reference:	KCP 5.1.2 (e)/10
Report:	RH-141452: HYDROLYSIS UNDER SIMULATED PROCESSING CONDITIONS, Longhi, D., 2019, report No. BPL-STUDY-18-000092, Doc. No. 638-008
Guideline(s):	OECD No. 111, CIPAC MT 75.3, SANCO/825/00 rev.8.1 (2010), OECD No. 507, ISO 6222:2001
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

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

Equipment

Instrument:	Agilent HPLC 1260 Infinity II
Column:	Poroshell 120 EC-C18, 4 µm, 4.6 x 100 mm;
Column temp.:	25°C

Mobile phase: A: 0.1 % v/v phosphoric acid in UPLC grade water (prepared dissolving a volume of 2.5 mL of phosphoric acid in 2.5 L of water);
B: 0.1 % v/v phosphoric acid in HPLC grade acetonitrile (prepared dissolving a volume of 2.5 mL of phosphoric acid in 2.5 L of acetonitrile);

Elution gradient:

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

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

Results and discussions

Table A 33: Summary of recovery experiments

Matrix	Fortification level (mg/L)	Accuracy and precision per level		Overall accuracy and precision	
		Accuracy (%) n=5	RSD (%) n=5	Mean accuracy (%) n=10	RSD (%) n=10
Buffer pH 4	1 (LOQ)	96.89	3.26	98.5	2.8
	10 (10 x LOQ)	100.2	0.4		
Buffer pH 5	1 (LOQ)	98.83	0.69	99.6	1.0
	10 (10 x LOQ)	100.3	0.6		
Buffer pH 6	1 (LOQ)	96.95	3.43	98.9	3.0
	10 (10 x LOQ)	100.8	0.4		

Accuracy and precision / repeatability

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

Linearity

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

Limit of quantification

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

Limit of detection

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

Matrix effects

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

Specificity

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

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

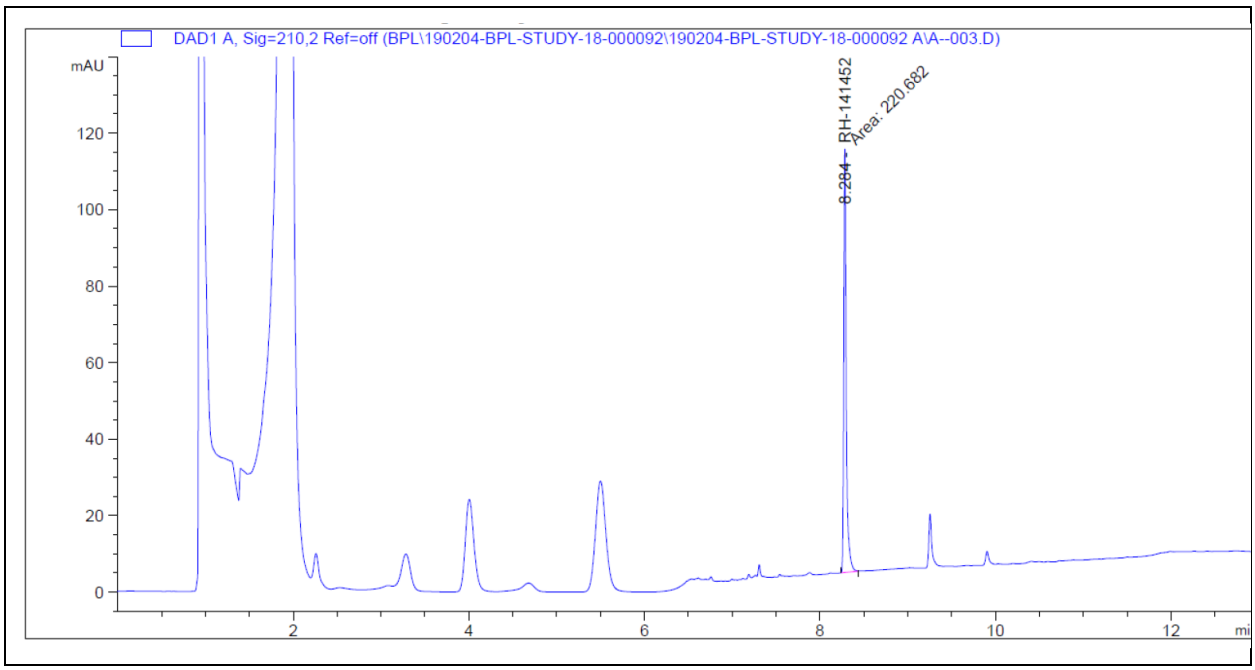
Storage of samples and storage stability

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

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

	RH-141452
Specificity	Mass spectrum provided. Blank value < 30% LOQ.
Calibration (type, number of data points)	Matrix matched standard calibration. 6 point calibration; r^2 >0.99; linear. Calibration data and calibration line equation presented in the study report. e.g.: y = 219.59082x + 5.35882
Calibration range	0.3024 – 15.12 mg/L
Assessment of matrix effects is presented	No. (Matrix matched standard clibration.)
Limit of determination/quantification	LOQ: 1 mg/L LOD: 0.1008 mg/L

The following figures show typical chromatograms and MS spectra.



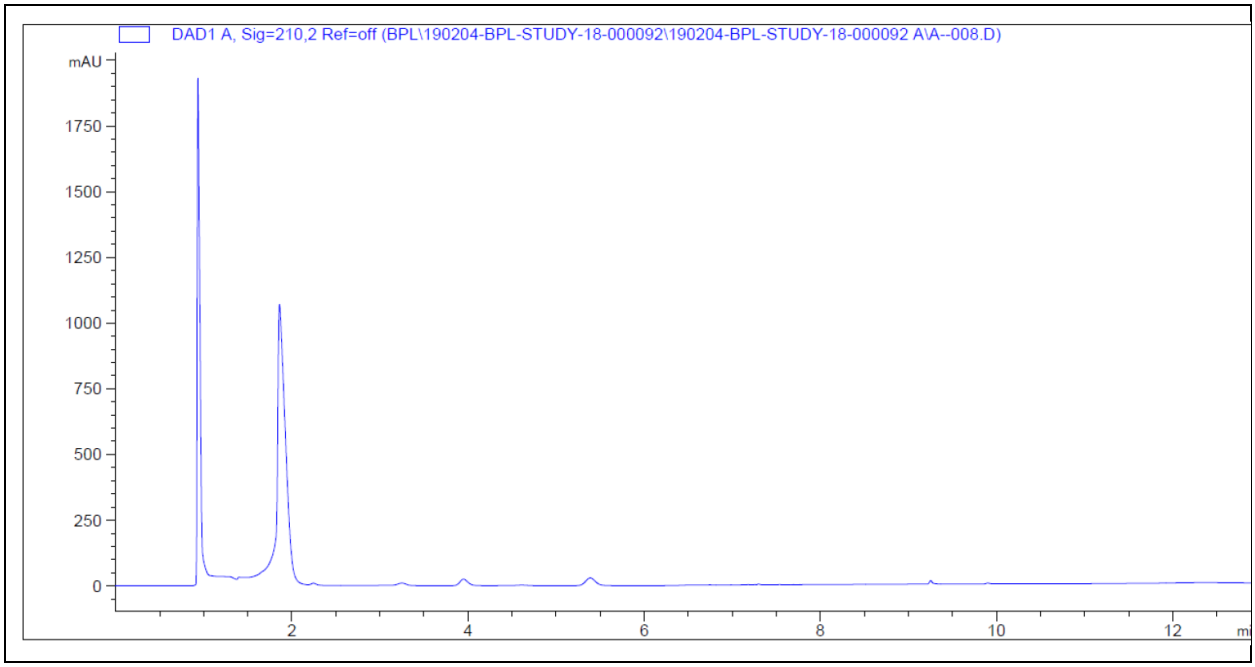
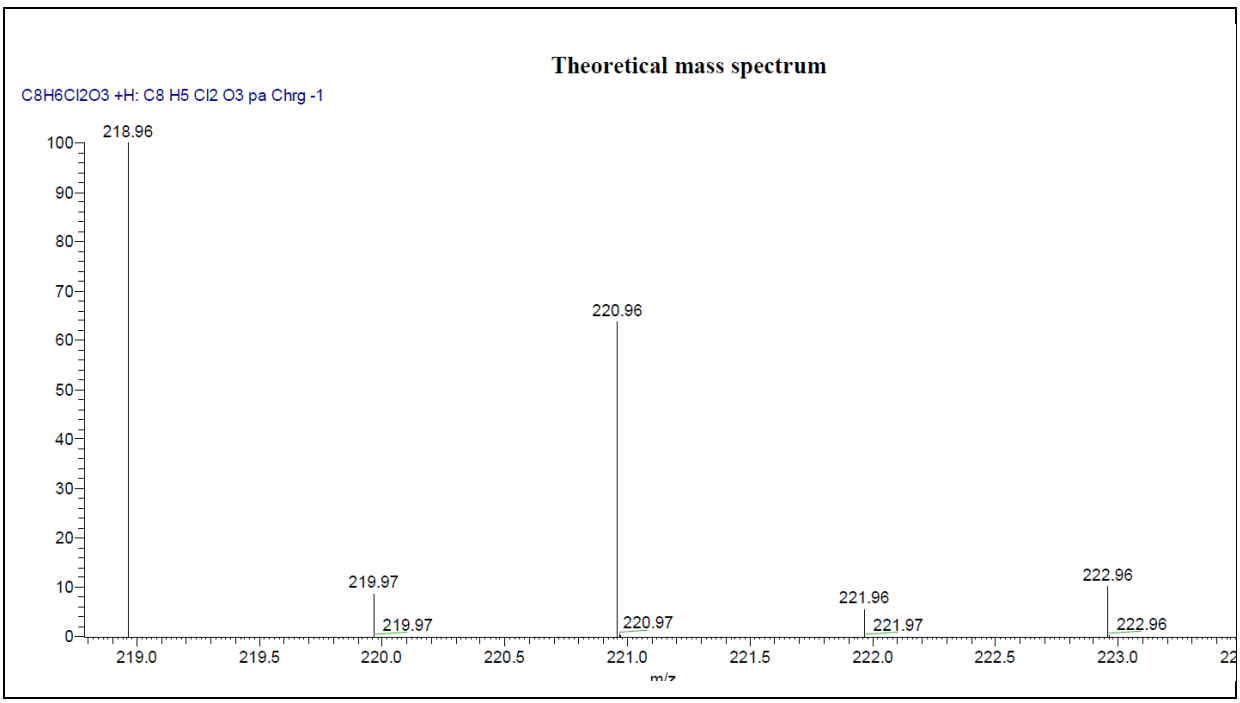


Figure A 4: **Representative UV DAD chromatograms showing RH-141452 standard at 1 mg/L (LOQ) in buffer solution of pH 4 (above) and matrix blank (below)**



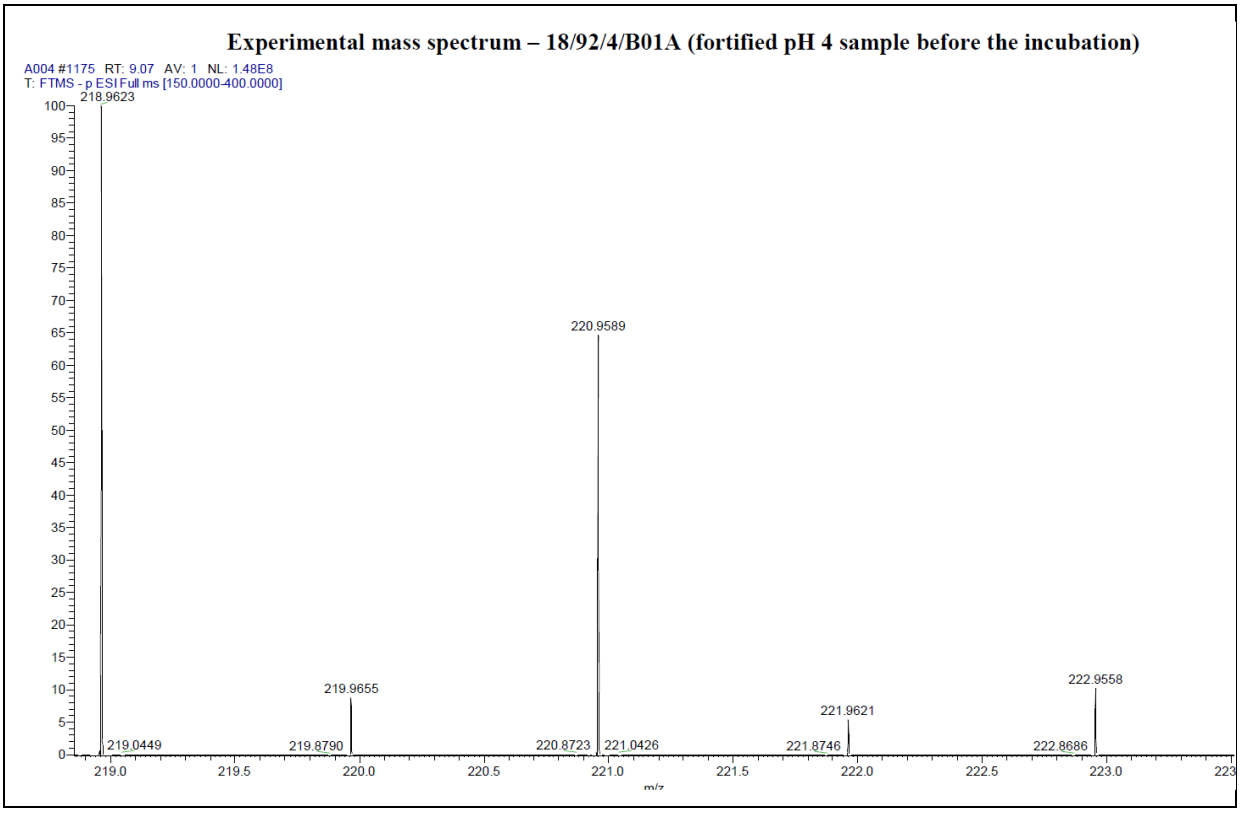


Figure A 5: Theoretical MS spectrum of RH-141452 (above) and spectrum of RH-141452 in a fortified buffer solution at pH 4 (below)

Conclusion

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

(Longhi D. 2019)

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The analytical method based on HPLC-DAD system acceptably validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) requirements. The LOQ is defined as the lowest concentration at which an acceptable recovery is obtained that corresponds to 1.012 mg/L of RH-141455 in all the buffers.*

Reference:	KCP 5.1.2 (e)/11
Report:	RH-141455: HYDROLYSIS UNDER SIMULATED PROCESSING CONDITIONS, Longhi, D., 2019, report No. BPL-STUDY-19-000009, Doc. No. 638-009
Guideline(s):	SANCO/825/00 rev.8.1 (2010), OECD No. 111, OECD No. 507, ISO 6222:2001, CIPAC MT 75.3
Deviations:	No

GLP: Yes
Acceptability: Yes

Materials and methods

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

Equipment

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

Gradient:

Time (min)	% A	% B
0	98	2
5	98	2
12	0	100
14	0	100

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

Results and discussions

Table A 35: Summary of recovery experiments

Matrix	Fortification Level (mg/L)	Accuracy and precision per level		Overall accuracy and precision	
		Accuracy (%) n=5	RSD (%) n=5	Mean Accuracy (%) n=10	RSD (%) n=10
Buffer pH 4	1 (LOQ)	99.13	3.62	99.29	2.46
	10 (10 x LOQ)	99.45	0.70		
Buffer pH 5	1 (LOQ)	100.5	1.5	98.60	2.8
	10 (10 x LOQ)	96.63	2.54		
Buffer pH 6	1 (LOQ)	91.08	0.39	94.99	4.73
	10 (10 x LOQ)	98.90	2.70		

Accuracy and precision / repeatability

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

Linearity

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

Limit of quantification

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

Limit of detection

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

Matrix effects

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

Specificity

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

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

Storage of samples and storage stability

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

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

	RH-141455
Specificity	Mass sprectrum provided. Blank values < 30% LOQ.
Calibration (type, number of data points)	Standard solution calibration. 6 point calibration range; r^2 >0.99; linear Calibration data and calibration line equation presented in the study report. e.g. y = 187.18322x + 36.79298
Calibration range	0.3036 – 15.18 mg/L
Assessment of matrix effects is presented	Yes. (Matrix effects were not significant (< 20%).)
Limit of determination/quantification	LOQ: 1 mg/L LOD: 0.1012 mg/L

The following figures show typical chromatograms and MS spectra.

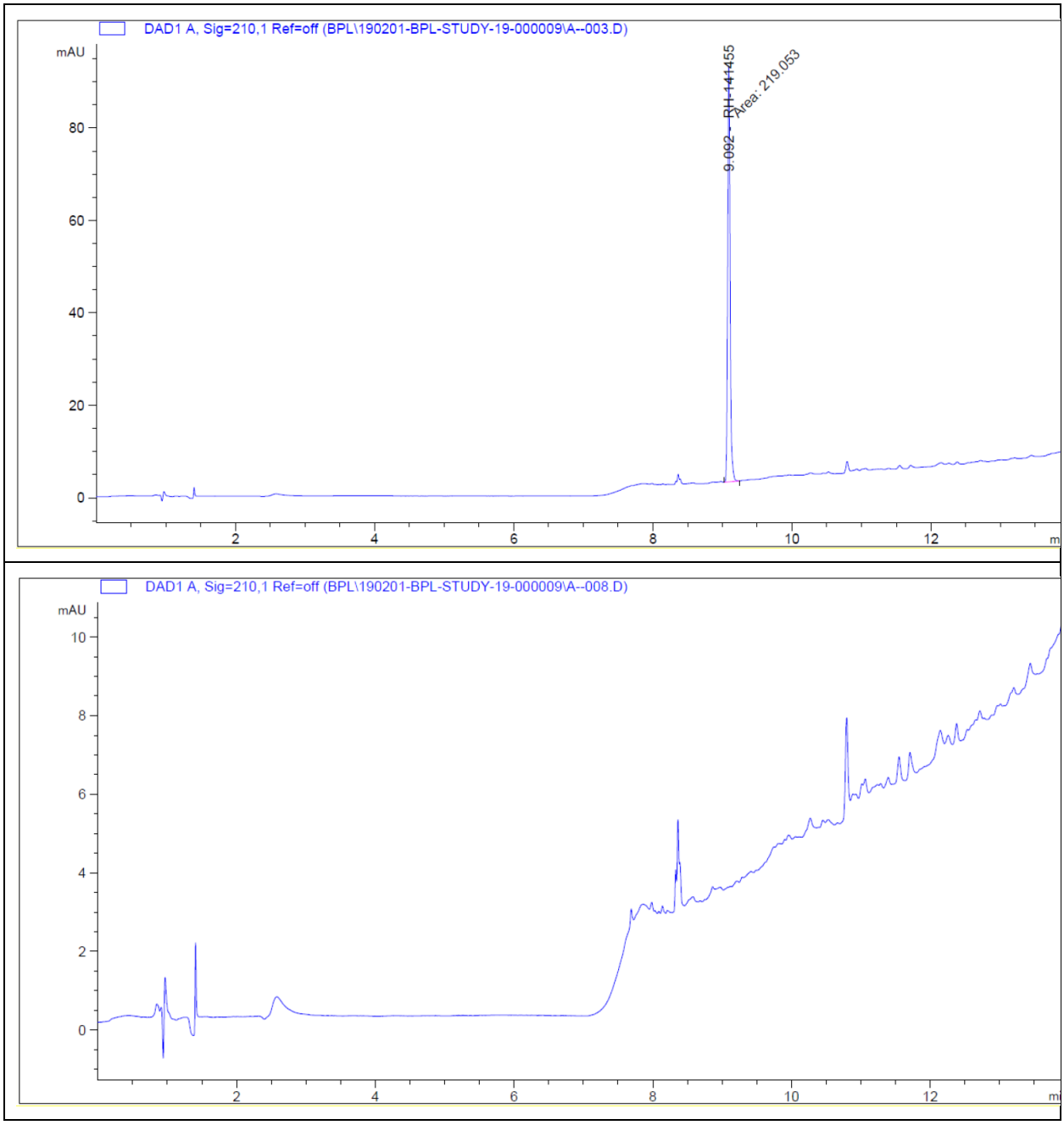


Figure A 6: **Representative UV DAD chromatograms showing RH-141455 standard at 1 mg/L (LOQ) in solution (above) and solvent blank (below)**

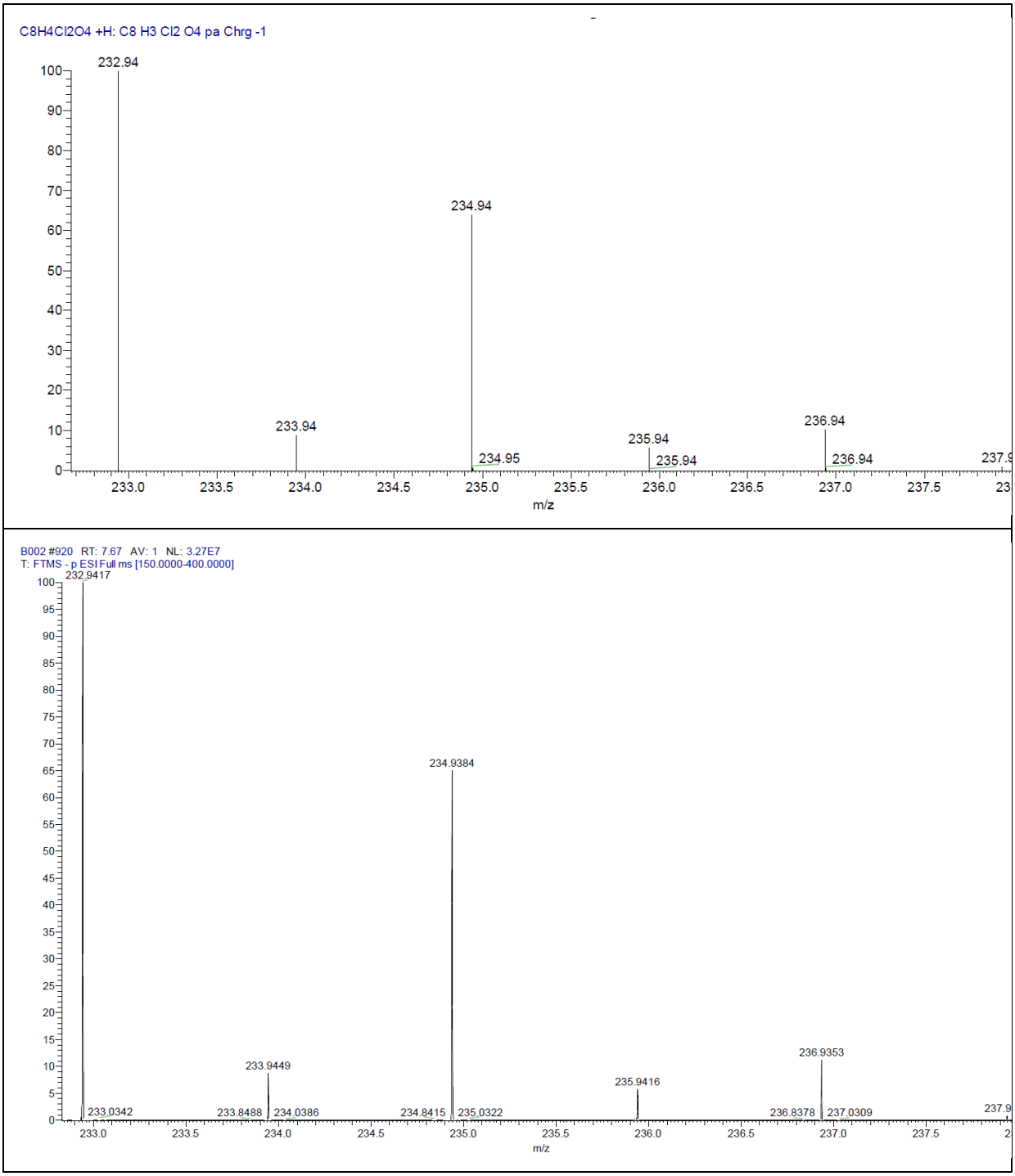


Figure A 7: Theoretical MS spectrum of RH-141455 (above) and spectrum of a RH-141455 in standard solution (acetonitrile) (below)

Conclusion

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

(Longhi D. 2019)

Methods used in ecotoxicity studies

Determination of Zoxamide and Phosphonic acid in water

Comments of zRMS:	The validation has been accepted. UHPLC-MS/MS analytical methods were applied. 2 transitions were monitored. The validity parameters were as required. It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (f)/01
Report:	VALIDATION OF THE ANALYTICAL METHODS (SANTE/2020/12830 REV.1) FOR THE DETERMINATION OF PHOSPHONIC ACID AND ZOXAMIDE IN AQUEOUS MATRIX SOLUTIONS WITH PRODUCT GWN-10616, Fifi, A.P., 2021, report No. BT233/21, Doc. No. 435-001
Guideline(s):	SANTE/2020/12830 REV.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

For determination of Zoxamide and phosphonic acid, samples of water (EPA medium) were diluted with water (EPA medium) and analysed by HPLC-MS/MS.

Chromatographic conditions – Zoxamide

System	HPLC-MS/MS
Column	Zorbax Eclipse Plus C18 1.8 µm, 50 x 2.1 mm
Mobile phase	Eluent A: Water with 0.1 % formic acid Eluent B: Acetonitrile
Monitored ions	336 > 187 and 336 > 159
Retention time	1.9 min

Chromatographic conditions – Phosphonic acid

System	HPLC-MS/MS
Column	AGILA Venusil HILIC 2.1x100 mm, 3 µm
Mobile phase	Eluent A: Water with 0.1 % formic acid Eluent B: Acetonitrile with 0.1 % formic acid
Monitored ions	81 > 79 and 81 > 63
Retention time	2.4 min

Results and discussions

The mean recovery at each fortification level were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 37: Recovery results from method validation of Zoxamide and Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water (EPA medium)	Zoxamide 336>187	0.2492 (n = 5)	102.54	2.36	-
		43.0937 (n = 5)	100.69	2.13	-

Matrix	Analyte	Fortification level (g/L) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water (EPA medium)	Zoxamide 336 > 159	0.1018 (n = 5)	101.84	2.70	-
Water (EPA medium)	Phosphonic acid 81 > 79	2.1438 (n = 5)	106.46	2.37	-
		352.1518 (n = 5)	106.11	5.90	-
Water (EPA medium)	Phosphonic acid 81 > 63	0.8373 (n = 5)	105.65	1.51	-

Table A 38: Characteristics for the method used for validation of Zoxamide and Phosphonic acid residues in Water (EPA medium)

	Zoxamide	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.0721 µg/L – 2.5746 µg/L Corresponding calibration range in mass ratio units for the sample: 0.0721 µg/L – 53.64 g/L	Accepted calibration range in concentration units: 0.5937 µg/L – 14.8437 µg/L Corresponding calibration range in mass ratio units for the sample: 0.5937 µg/L – 530.1321 g/L
Assessment of matrix effects is presented	Due to the high dilution factors matrix effects can be excluded. Calibration was carried out in marix-matched standards, so potential marix effects were compensated in any case.	Due to the high dilution factors matrix effects can be excluded. Calibration was carried out in marix-matched standards, so potential marix effects were compensated in any case.
Limit of determination/quantification	0.2492 g/L	2.1438g/L

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide and Phosphonic acid in water (EPA medium).

Determination of Zoxamide and phosphonic acid in sugar solutions

Comments of zRMS:	The validation has been accepted. UHPLC-MS/MS analytical methods were applied. 2 transitions were monitored. The validity parameters were as required. It could be concluded that the method is applicable for the intended purpose.

Reference:	KCP 5.1.2 (f)/02
Report:	VALIDATION OF THE ANALYTICAL METHODS (SANTE/2020/12830 REV.1) FOR THE DETERMINATION OF PHOSPHONIC ACID AND ZOXAMIDE IN SUGAR FEEDING SOLUTIONS WITH PRODUCT GWN-10616, Fifi, A.P., 2021, report No. BT234/21, Doc. No. 437-001
Guideline(s):	SANTE/2020/12830 REV.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

For determination of Zoxamide, samples of sugar feeding solution were diluted with acetonitrile and centrifuged. After further dilution, analysis was carried out by HPLC-MS/MS.
For determination of phosphonic acid, samples of sugar feeding solution were diluted with water. An aliquot was added to a Quechers kit (centrifuge tube filled with MgSO4 + PSA) and centrifuged. The supernatant was diluted before analysis by HPLC-MS/MS.

Chromatographic conditions – Zoxamide

System	HPLC-MS/MS
Column	Zorbax Eclipse Plus C18 1.8 µm, 50 x 2.1 mm
Mobile phase	Eluent A: Water with 0.1 % formic acid Eluent B: Acetonitrile
Monitored ions	336 > 187 and 336 > 159
Retention time	1.9 min

Chromatographic conditions – Phosphonic acid

System	HPLC-MS/MS
Column	AGILA Venusil HILIC 2.1x100 mm, 3 µm
Mobile phase	Eluent A: Water with 0.1 % formic acid Eluent B: Acetonitrile with 0.1 % formic acid
Monitored ions	81 > 79 and 81 > 63
Retention time	2.4 min

Results and discussions

The mean recovery at each fortification level were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 39: Recovery results from method validation of Zoxamide and Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (g/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Sugar feeding solution	Zoxamide 336>187	0.1018 (n = 5)	104.14	2.04	-
		3.6307 (n = 5)	89.26	2.27	-

Matrix	Analyte	Fortification level (g/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Sugar feeding solution	Zoxamide 336 > 159	0.1018 (n = 5)	101.94	6.33	-
Sugar feeding solution	Phosphonic acid 81 > 79	0.8373 (n = 5)	98.11	4.77	-
		29.8291 (n = 5)	98.79	0.87	-
Sugar feeding solution	Phosphonic acid 81 > 63	0.8373 (n = 5)	98.22	7.50	-

Table A 40: Characteristics for the method used for validation of Zoxamide and Phosphonic acid residues in sugar feeding solution

	Zoxamide	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.0733 µg/L – 2.6163 µg/L Corresponding calibration range in mass ratio units for the sample: 0.0293 g/kg – 4.7569 g/kg	Accepted calibration range in concentration units: 0.6183 µg/L – 15.4574 µg/L Corresponding calibration range in mass ratio units for the sample: 0.2473 g/kg – 44.164 g/kg
Assessment of matrix effects is presented	Yes	Yes
Limit of determination/quantification	0.1018 g/kg	0.8373 g/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide and Phosphonic acid in sugar feeding solution.

Determination of Zoxamide and phosphonic acid in sugar feeding solutions and water

Comments of zRMS:	The validation has been accepted. The analysis was performed by LC/MS-MS following the validated method. 2 transitions were monitored. The validity parameters were as required. It could be concluded that the analytical method was suitable to determine zoxamide and phosphonic acid in the feeding solutions.

Reference:	KCP 5.1.2 (f)/03
Report:	CHRONIC ORAL EFFECTS OF GOW F716 (GWN-10616) TO ADULT WORKER HONEYBEES APIS MELLIFERA L. 10-DAY FEEDING LABORATORY TEST, Colli, M., 2021, report No. BT147/17, Doc. No. 832-002
Guideline(s):	OECD No. 245 (2017)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were diluted with water and analysed by HPLC-MS/MS.

Chromatographic conditions – Zoxamide

System	HPLC-MS/MS
Column	Zorbax Eclipse Plus C18 1.8 µm, 50 x 2.1 mm
Mobile phase	Solvent A Water acidifed 0.1% formic acid Solvent B Acetonitrile Ratio A/B = 40/60
Monitored ions	336 > 187 and 336 > 159
Retention time	2.5 min

Chromatographic conditions – Phosphonic acid

System	HPLC-MS/MS
Column	Phenomex Gemini NX-C18 – 110 Å, 150 x 3 mm, 3 µm
Mobile phase	Solvent A Water acidified 0.5% formic acid + 10% MeOH Solvent B Acetonitrile Ratio A/B = 90/10
Monitored ions	81 > 79 and 81 > 63
Retention time	1.8 min

Results and discussions

The mean recovery at each fortification level were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 41: Recovery results from method validation of Zoxamide and Phosphonic acid using the analytical method

Matrix	Analyte	Fortification level (µg/L for water; mg/kg for feeding solution) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water	Zoxamide 336>187	1.085 (n = 5)	89.0	4.57	-
		10.85 (n = 5)	95.08	2.40	-
Water	Zoxamide 336 > 159	0.1018 (n = 5)	88.17	3.80	-
Sugar feeding solution	Zoxamide 336>187	5.43 (n = 5)	90.17	2.26	
		54.26 (n = 5)	88.33	0.324	
Sugar feeding solution	Zoxamide 336 > 159	5.43 (n = 5)	90.29	1.64	
Water	Phosphonic acid 81 > 79	834460 (n = 5)	88.31	1.65	-
		8344620 (n = 5)	88.77	1.78	-
Water	Phosphonic acid 81 > 63	834460 (n = 5)	88.13	3.08	-
Sugar feeding solution	Phosphonic acid 81 > 79	43.37 (n = 5)	90.53	3.41	-
		433.70 (n = 5)	90.75	4.34	-
Sugar feeding solution	Phosphonic acid 81 > 63	43.37 (n = 5)	88.33	3.24	-

Table A 42: Characteristics for the method used for validation of Zoxamide and Phosphonic acid residues in sugar feeding solution and water

	Zoxamide	Phosphonic acid
Specificity	Mass spectrum is provided. blank value < 30 % LOQ	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.493 to 14.798 µg/L Corresponding calibration range in mass ratio units for the sample: 0.493 to 14.798 µg/L for water 1.2325 to 369.95 mg/kg for sugar feeding solution based on a dilution factor of 500	Accepted calibration range in concentration units: 223.10 to 892.40 µg/L Corresponding calibration range in mass ratio units for the sample: 446.2 – 17848 mg/L in water based on dilution factors of 2000 and 20000 2.231 – 892.4 mg/kg based on dilution factors of 10 and 100

	Zoxamide	Phosphonic acid
Assessment of matrix effects is presented	Due to the high dilution factors matrix effects can be excluded.	Due to the high dilution factors matrix effects can be excluded.
Limit of determination/quantification	1.085 µg/L in water 5.43 mg/kg in sugar feeding solution	834.46 mg/L in water 43.37 mg/kg in sugar feeding solution

Compliance with SANTE/2020/12830 rev. 2

According to the new guideline, linearity should preferably be demonstrated by residuals. In the report summarized above, the peak areas are included. Based on the determined peak areas of the individual calibration standards and the presented calibration graph, it can be concluded that the residuals would be randomly distributed. Therefore, linearity is demonstrated. According to the new guideline, linearity should extend from 30% of the LOQ to +20% of the highest level. As the method was validated according to SANCO/3029/99 rev. 4, the range covers 80% of the LOQ. As all other validation parameters are in compliance with the new guideline, this is considered to have no adverse effect on the method validation. In addition, the minimum validation data for methods validated before the implementation of SANTE/2020/12830 rev. 2 are fulfilled in any case.

Conclusion

The method is valid and acceptable for the determination of Zoxamide and Phosphonic acid in sugar feeding solution and water.

Methods for metabolite RH-163353

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The LC-TOF/MS method was acceptably validated for the analyte RH-163353 in samples. LOQ is 0.001 mg/L for fish and 0.1 mg/L for Daphnia, mysid and alga.*

Reference:	KCP 5.1.2 (f)/04
Report	Goodband, T., 2020: RH-163353: Fish acute toxicity test - Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202385, GLP, Not published
Guideline(s):	SANCO 3029/99 rev 4 (2000)
Deviations:	No
GLP	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/05
Report	Jarrom, R., 2020: RH-163353 Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202386, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	A temperature deviation was noted during the range finding and definitive test because the continuous temperature vessel variation was >2°C (range finding test = 2.3°C; definitive test = 2.1°C). This slight deviation (protocol specification = within 2°C) was not considered to have had an impact on the integrity of the study.
GLP	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/06
Report	Jarrom, R., 2020: RH-163353: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202387, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4 (2000)

Deviations:	The measured pH of the 100 mg/L solution at test initiation was 7.37 (0.13 outside of the specified range of 7.5 – 8.5), pH adjustment was not made. This protocol deviation has no impact on the integrity of the study as no mortality was seen in the 100 mg/L replicates, demonstrating no negative effects on the test organisms.
GLP	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/07
Report	Jarrom, R., 2020: RH-163353: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202388, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	Additional light measurements were taken at 24 and 48-hours to ensure the light remained within the correct ranges. The maximum temperature recorded during the test was 24.6°C and therefore, this exceeded the protocol range (21–24°C). This temperature deviation occurred within the first 45-hour of the test. As the test temperatures were measured at approximately 24-hour intervals it is not possible to confirm the exact length of the out of specification period, however, the temperature was out of specification (actual temperature = 24.4°C) when recorded at ca 21-hours but was back in specification (actual temperature = 23.4°C) when recorded at 45-hours. As the temperature remained within 2°C, the algae growth was good and achieved all the control validity criteria, this protocol deviation was not considered to have an impact on the integrity of the study.
GLP	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202385-01V (and updated version SMV 3202385-02V to include storage stability data), SMV 3202386-01V (and updated version SMV 3202386-02V to include storage stability data), SMV 3202387-01V (later updated SMV 3202387-02V to add the stability information) and SMV 3202388-01V (and updated version SMV 3202388-02V to include storage stability data) were used to determine the concentration of the analyte RH-163353 in samples of test media from fish acute, *Daphnia* acute, mysid and alga growth studies, respectively. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

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

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

Equipment (LC-TOF/MS Analysis)

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

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

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

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

Equipment for enantiomeric ratio analysis

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

Results and discussions

Table A 43: Summary of recovery experiments in the fish study

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

Table A 44: Summary of recovery experiments in the daphnid study

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

Table A 45: Summary of recovery experiments in the mysid study

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

Table A 46: Summary of recovery experiments in the alga study

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

Accuracy and precision/repeatability

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

Linearity

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

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

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

Limit of quantification

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

Matrix effects

Matrix-matched standards were used.

Specificity

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

Storage stability

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

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

Table A 47: Characteristics of the analytical method validations for RH-163353 in aquatic media

	RH-163353
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-point calcibrations, linear with 1/x weighting; r > 0.99. Calibration data and calibration line equations presented in the study reports. Example equations are : y = 9.93512e5 x^2 + 5.90123e5x + 17.23614 (fish), r = 99965 y = 7.34870e6 x^2 + 6.92065e6x + 66.52811 (daphnia), r = 99987 y = 8.19738e6 x + 3243.99688(mysid), r = 99210 y = 1.64705e6 x + +510.56044 (alga), r = 99451
Calibration range	0.0001 – 0.1 µg/mL (fish, mysid, alga) 0.001 – 0.5 µg/mL (*Daphnia*)
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ)	0.001 mg/L (fish) 0.1 mg/L (*Daphnia*, mysid, alga)

The following figures show typical chromatograms.

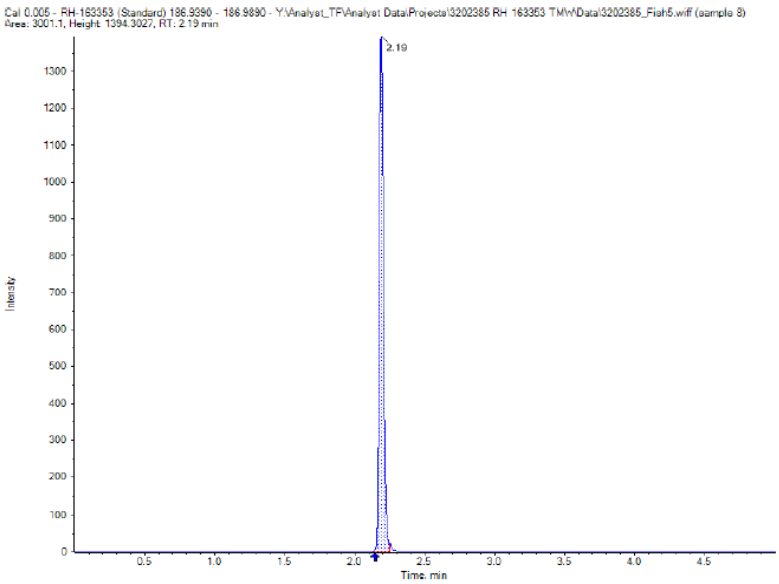


Figure A 8: **Chromatogram of RH-163353 (sum of isomers)**

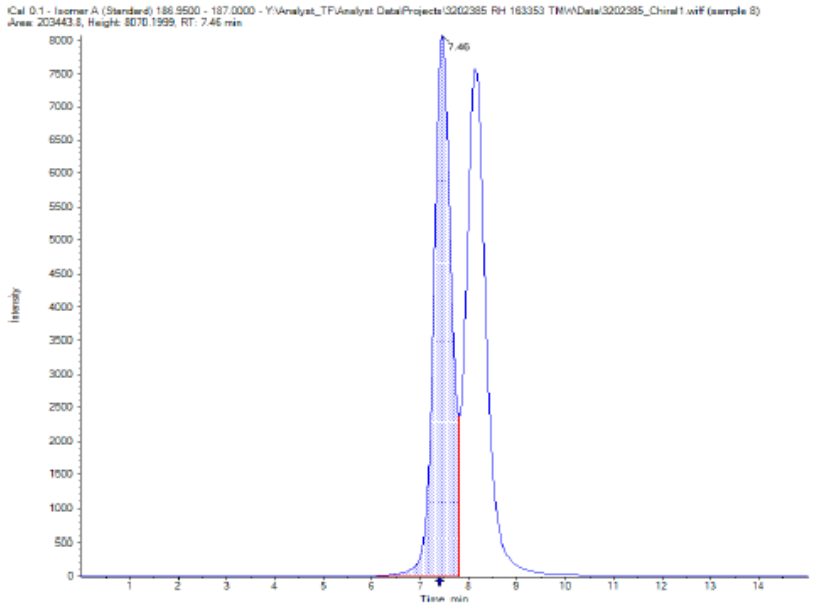


Figure A 9: **Chromatogram of Isomer A**

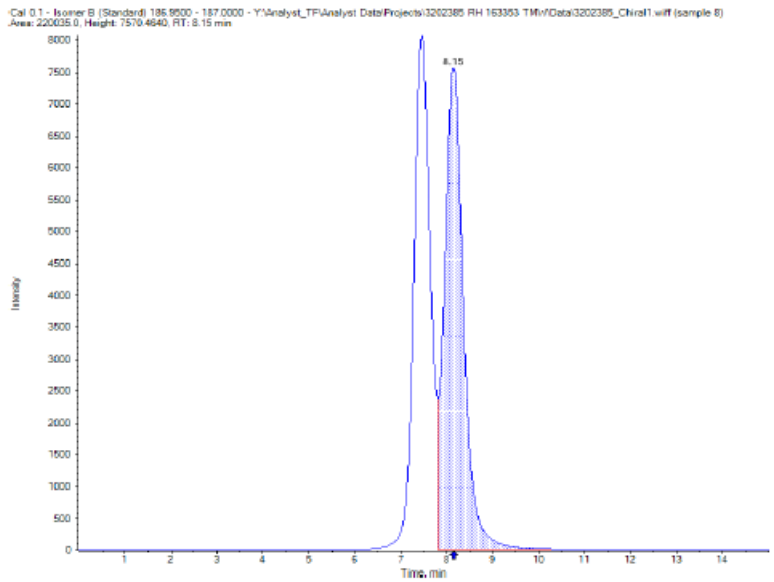


Figure A 10: **Chromatogram of Isomer B**

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

Table A 48: Enantiomeric ratio of 96-hour sample in fish medium

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

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

Table A 49: Enantiomeric ratio of 48-hour samples in *Daphnia* medium

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

Conclusion

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

(Goodband T. 2020, Jarrom R. 2020 a,b,c)

Methods for metabolite RH-141455

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The analysis by liquid chromatography-time of flight mass spectrometry was acceptably validated for the analyte RH-141455 in samples. The LOQ are 0.1 mg/L (fish and Daphnia) and 0.25 mg/L (mysid).*

Reference:	KCP 5.1.2 (f)/08
Report	Goodband, T., 2020: RH-141455: Fish acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202716, GLP, Not published
Guideline(s):	SANCO 3029/99 rev 4 (2000)
Deviations:	No
GLP	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/09
Report	Hugill, E., 2019: RH-141455: Acute toxicity to *Daphnia magna* Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202380, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	There was no confirmation in the range finding data that the vitamin stock was added to the Elendt M4 media by error. This protocol deviation has no impact on the integrity of the study because the main test was performed without deviation and the immobilisation at all treatments was ≤ 10% - indicating that there was no impact on *Daphnia* if the vitamin stock was not added. A temperature deviation was noted during the test because the 100 mg/L treatment vessel temperature deviated by 2.2°C from the initial vessel temperature. This slight deviation (protocol specification = within 2°C) was not considered to have had an impact on the integrity of the study, as there was not test substance immobilisation during the test.
GLP	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/10
Report	Hugill, E., 2020: RH-141455: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202381, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	Only some minor deviations during the range-finder, which are considered to be not relevant for the integrity of the (main) study results.
GLP	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202716-01V (and updated version SMV 3202716-02V to include storage stability data), SMV 3202380-02V, and SMV 3202381-01V (and updated version SMV 3202381-03V to include storage stability data) were used to determine the concentration of the analyte RH-141455 in samples of test media from acute fish, *Daphnia* and mysid studies, respectively. This method describes the analysis in Elendt M4 medium, treated mains water and in brackish water. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

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

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

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

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

Equipment for LC-TOF/MS analysis

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

Mobile phase: A: 0.1% formic acid in water (LC-MS grade)
B: 1% formic acid in acetonitrile (LC-MS grade)
Fish acute toxicity test

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

Acute toxicity to *Daphnia*

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

Mysid acute toxicity test

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

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

Results and discussions

Table A 50: Summary of recovery experiments - fish

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

Table A 51: Summary of recovery experiments - *Daphnia*

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

Table A 52: Summary of recovery experiments - mysid

Brackish water	0.25 µg/mL	1 µg/mL	150 µgm/L	Overall
Mean Recovery (%)	90.4	93.9	113	98.2
CV (%)	8.18	5.96	5.54	11.8

Accuracy and precision / repeatability

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

Linearity

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

Limit of quantification

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

Matrix effects

Matrix-matched standards were used.

Specificity

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

Storage stability

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

Table A 53: Characteristics of the analytical method validation for the determination of RH-141455 in aquatic samples

	RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r >0.99. Individual calibration data and calibration line equations presented in the study report Example equations are :

	RH-141455
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
	y = 6.84008e4x^2 + 6.64717e5x + 304.15146 (fish), r = 0.99614 y = 1.83184e5x + 186.22482 (daphnid), r = 0.99483 y = 7689.58330x^2 + 7837.03325x + 11.55719 (mysid), r = 0.99962
Calibration range	0.01 – 1 µg/mL (fish) 0.002 – 0.5 µg/mL (*Daphnia*) 0.005 – 0.5 µg/mL (mysid)
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of quantification (LOQ)	0.1 mg/L (fish and *Daphnia*) 0.25 µg/mL (mysid)

The following figure shows a typical chromatogram.

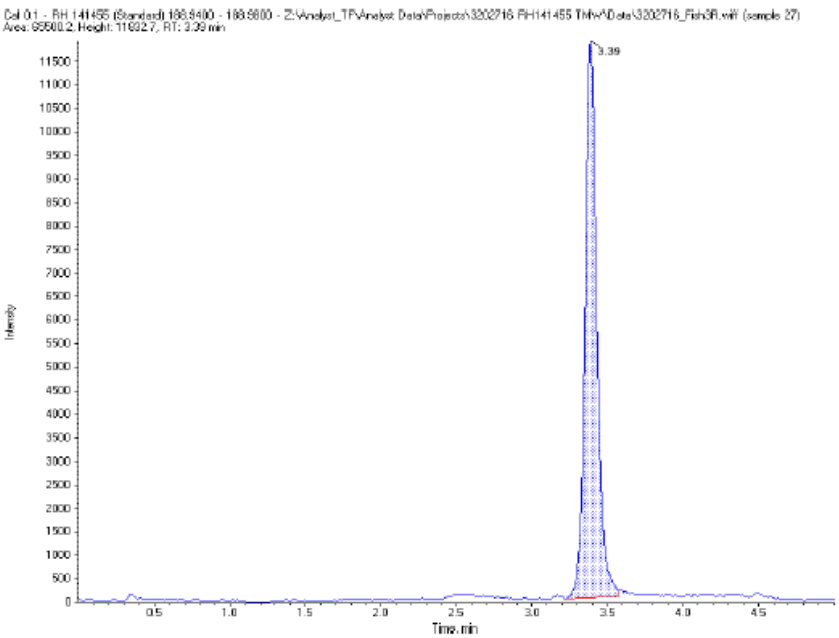


Figure A 11: **Chromatogram of RH-141455**

Conclusion

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

(Goodband T. 2020, Hugill E. 2019, Hugill E. 2020)

Methods for metabolite RH-127450

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia on the studies below was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *Analysis by LC-TOF/MS and LC-TQMS methods meets the criteria of specificity, linearity, repeatability and accuracy. The mean overall recovery in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable. The LOQ of applied procedure is 0.01 mg/L (alga, mysid) and 0.001 µg/L (fish).*

Reference:	KCP 5.1.2 (f)/11
Report	Goodband, T., 2020: RH-127450: Fish, acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202373, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/12
Report	Hugill, E., 2020: RH-127450: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202374, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	During the range finding test, the salinity of the control and highest concentration (100% saturated solution) exceed the ± 1 ppt protocol requirement, as these were measured to be 22 and 18 ppt, respectively at the start of the test. This was not identified at the time in error, however, has no impact as the protocol requirement was tighter than the guideline (± 2 ppt). Additional Dissolved oxygen concentrations were taken from the treatment vessels where 100% mortality was noted at the Study Director's direction. These were taken to confirm that the high mortality was not related to low oxygen levels, as mysids are very sensitive to oxygen levels. However, this protocol deviation has no impact on the integrity of the study. The protocol only required statistical analysis to be conducted on the 48 and 96-hour results, but should have included the conduct of statistical analysis on the 24 and 72-hour data, as this is a requirement of the OCSPP guideline.
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/13
Report	Hugill, E., 2020: RH-127450: Inhibition of growth to the alga *Raphidocelis subcapitata* Gowan Crop Protection Ltd., UK Smithers ESG Ltd., Report No. 3202375, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	An initial definitive test was conducted at nominal concentrations of 1, 3.2, 10, 32 and 100% saturated solution. However, it was necessary to repeat the main test due to an equipment failure (lux meter) and since, by error, 24- and 48-hour samples were not taken for analysis. The appearance of the test item in the test media was only observed at the start and at the end of the test. This deviation has no impact on the integrity of the study as the non-inoculated media observation at 72-hours showed the test substance to be in solution.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202373-01V (and updated version SMV 3202373-02V to include analysis and validation using a liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system and storage stability data), SMV 3202374-01V (and later updated version SMV 3202374-02V to add storage stability data) and SMV 3202375-01V (and later updated version SMV 3202337-02V to add storage stability data) were used to determine the concentration of the analyte RH-127450 in samples of test media from acute fish, acute mysid and alga growth studies, respectively. The method describes the analysis of RH-127450 in mains water, brackish water and OECD medium. The procedures have been validated for each test medium according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

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

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

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

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

Equipment for LC-TOF/MS analysis

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

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

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

Equipment for LC-TQMS analysis

Instrument: API 5000 TQMS coupled to Shimadzu SIL-30ACMP Quaternary HPLC (mysid, fish)
Agilent 1100 HPLC system with UV detector or Quaternary HPLC
Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1 mm
Mobile phase: A: 0.1% formic acid in water (LC-MS grade)
B: 0.1% formic acid in acetonitrile (LC-MS grade)

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

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

Results and discussions

Table A 54: Summary of recovery experiments - mysid

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

CV Coefficient of variation

Table A 55: Summary of recovery experiments – alga

OECD Medium	0.01 µg/mL	0.5 µg/mL	12 µg/mL	Overall
Mean Recovery (%)	106	101	97.3	101
CV (%)	3.80	5.55	4.82	5.68

CV Coefficient of variation

Table A 56: Summary of recovery experiments – fish

Main water	0.01 µg/mL	1µg/mL	150 µg/mL	Overall
	LC-TOF/MS			
Mean Recovery (%)	112	107	83.1	101
CV (%)	4.0	1.4	5.4	14
	LC-TQMS			
Mean Recovery (%)	101	99.6	95.9	98.8
CV (%)	3.4	4.0	2.2	3.7

CV Coefficient of variation

Accuracy and precision / epeatability

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

Linearity

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

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

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

Limit of quantification

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

Matrix effects

Matrix-matched standards were used.

Specificity

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

Storage stability

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

Table A 57: Characteristics of the analytical method validation for the determination of RH-127450 in aquatic samples

	RH-127450
Specificity	Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r > 0.99. Individual calibration data and calibration line equations presented in the study report. Example equations are : y = 1.49e + 007 x^2 + 2.8e x + 80.9 (fish), r = 0.9997 y = 2.33e + 008 x^2 + 4.75e + 007 x + 5.53e + 003 (mysid), r = 0.9918 y = 3.09941e6 x + 2447.08707 (alga), r = 0.99804
Calibration range	0.0001 – 0.1 µg/mL (mysid, fish) 0.001 – 0.1 µg/mL (alga)
Assessment of matrix effects is presented	No (matrix matched standard solutions).
Limit of quantification (LOQ)	0.001 mg/L (fish) 0.01 mg/L (mysid and alga)

The following figure shows a typical chromatogram.

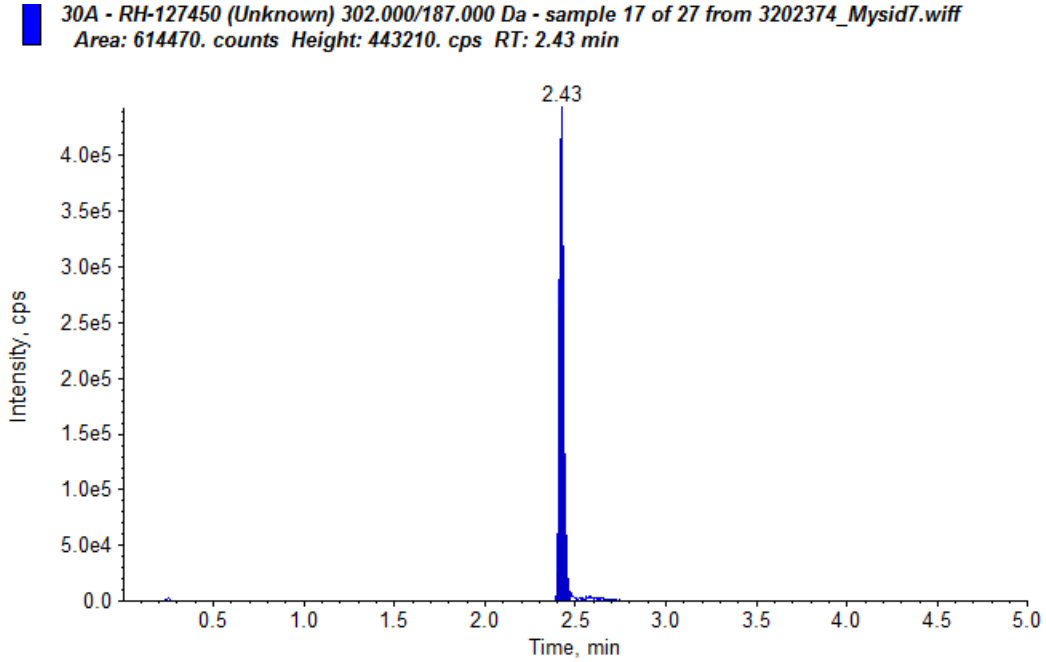


Figure A 12: **Chromatogram of RH-127450 (6.25% saturated solution test sample at 96 hours; dilution factor = 31.25)**

Conclusion

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

Method for RH-139432

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) or triple quadrupole mass spectrometry (LC-TQMS) analysis methods were acceptably validated and considered specific to RH-139432. The LOQ of the procedure is 0.1 µg/mL.*

Reference:	KCP 5.1.2 (f)/14
Report	Hugill, E., 2020: RH-139432: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202398, GLP, Not published
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	The test media was prepared using reverse osmosis water rather than deionised water as per the definitive protocol. This protocol deviation has no impact on the integrity of the study because reverse osmosis water is acceptable according to OCSPP 850.1035. The study protocol requested statistical analysis only for the 48 and 96-hour results. However, according to OCSPP the protocol should also have been mentioned statistical evaluation of the 24 and 72-hour data, what has finally been performed and reported.
GLP:	Yes
Acceptability:	Yes

Materials and methods

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

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

Equipment for LC-TOF/MS analysis

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

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

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

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

Equipment for LC-TQMS analysis

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

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

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

Results and discussions

Table A 58: Summary of recovery experiments

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

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

CV Coefficient of variation

Accuracy and precision/repeatability

The accuracy and repeatability/precision of the procedure were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ.

Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

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

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

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

Limit of quantification

The LOQ of the procedure is 0.1 mg/L.

Matrix effects

Matrix-matched standards were used.

Specificity

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

Storage stability

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

Table A 59: Characteristics of the analytical method for the determination of RH-139432 in aquatic media

	RH-139432
Specificity	LC-TOF/MS and LC-TQMS are regarded as highly specific. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix-matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r > 0.99. Individual calibration data and calibration line equations presented in the study report. Example equation : y = 1.34e + 006 x + 1.38, r = 0.9993
Calibration range	0.0001 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix-matched standard solutions).
Limit of quantification (LOQ)	LOQ: 0.1 mg/L

The following figure shows a typical chromatogram.

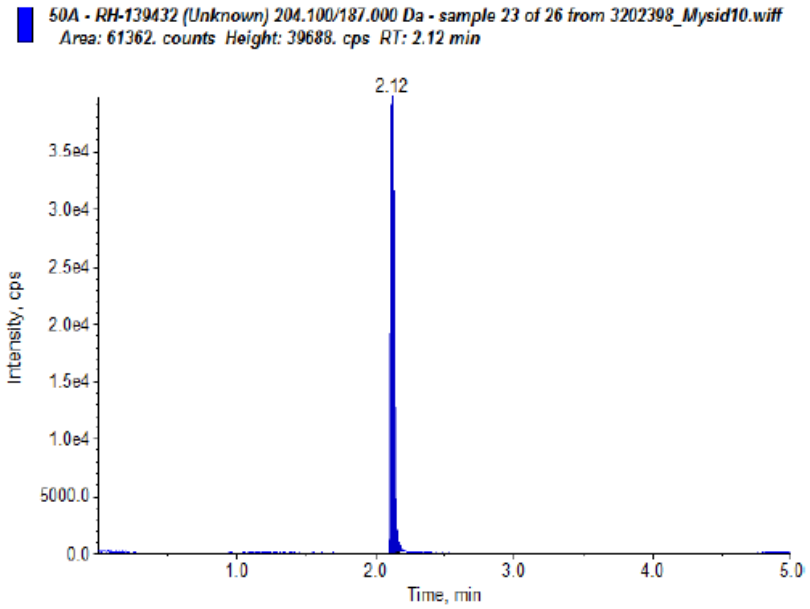


Figure A 13: **Chromatogram of RH-139432**

Conclusion

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

(Hugill E. 2020)

Method for RH-24549

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) or triple quad mass spectrometry (LC-TQMS/MS) methods acceptably validated according to SANTE/2020/12830, Rev.1, February 2021 guidance. The limit of quantification (LOQ) was 0.1 mg/L.*

Reference:	KCP 5.1.2 (f)/15
Report	Hugill, E., 2020: RH-24549: Mysid acute toxicity test Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202394, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4
Deviations:	The test media was prepared using reverse osmosis water rather than deionised water as per the definitive protocol. This protocol deviation has no impact on the integrity of the study because reverse osmosis water is acceptable according to OCSPP 850.1035. The salinity in the highest concentration dropped to 18 ppt (protocol requirement = 20±1 ppt during the test). This protocol deviation has no impact on the integrity of the study because salinity at 20±2 ppt is acceptable according to OCSPP 850.1035. The protocol required statistical analysis to be conducted on the 48 and 96-hour results. However, it should have included additional statistical analysis on the 24 and 72-hour data. This protocol deviation has no impact on the integrity of the study since the necessary calculations have been performed. A protocol deviation occurred at the highest concentration, because at 0-hours the pH in one test vessel was below pH 7.5 (allowed range: pH 7.5-8.5). This pH deviation was considered to have not an impact on the integrity of the study, because the 50% lethal effects concentration was between 25-50 mg/L where the pH measurements were within the acceptable range.
GLP:	Yes
Acceptability:	Yes

Materials and methods

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

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

TQMS) system.

Equipment for LC-TOF/MS analysis

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

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

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	10 µL
Retention time:	2.16 min approx.
Analysis time:	5 min
Ionisation:	Electrospray (ESI), negative
Ion mode:	m/z 202.97 → m/z 158.953-159.003

Equipment for LC-TQMS analysis

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

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

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

Results and discussions

Table A 60: Summary of recovery experiments

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

CV Coefficient of variation

Accuracy and precision/ repeatability

The accuracy and repeatability precision of the procedures were determined by fortifying aliquots of media in quintuplet at three different concentration levels. The results for accuracy and precision showed RSD values < 10 % for all spiking levels and per medium. Recovery values were in the required range of 70-110%. Untreated samples showed residues < LOQ.

Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

The detector response of the LC-TOF/MS and LC-TQMS analysis for RH-24549 was determined over the concentration range 0.0005 – 0.1 µg/mL using calibration solutions prepared in diluent. At least 6-point calibrations were performed over the concentration ranges. The correlation coefficient (r) values were all greater than 0.99. The equations of the calibration graphs were linear with 1/x weighting. All test samples were within the appropriate range of calibration standards.

Limit of quantification

The LOQ of the procedure is 0.1 mg/L.

Matrix effects

Matrix-matched standards were used.

Specificity

Liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) or triple quad mass spectrometry (LC-TQMS/MS) were considered specific as the analysis of the treated media exhibited no response exceeding 30% of the proposed LOQs.

Storage stability

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

Table A 61: Characteristics of the analytical method for the determination of RH-24549 in aquatic medium

	RH-24549
Specificity	LC-TOF/MS and LC-TQMS/MS are regarded as highly specific. Mass spectrum is provided. Blank value < 30 % LOQ.

Calibration (type, number of data points)	Matrix matched standard calibration. At least 6-points calibration curves; linear with 1/x weighting; r > 0.99. Individual calibration data and calibration line equations presented in the study report Example equation : y = 2.03e + 006 x + 631, r = 0.9992
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix-matched standard calibration).
Limit of quantification (LOQ)	LOQ: 0.1 mg/L

The following figure shows a typical chromatogram.

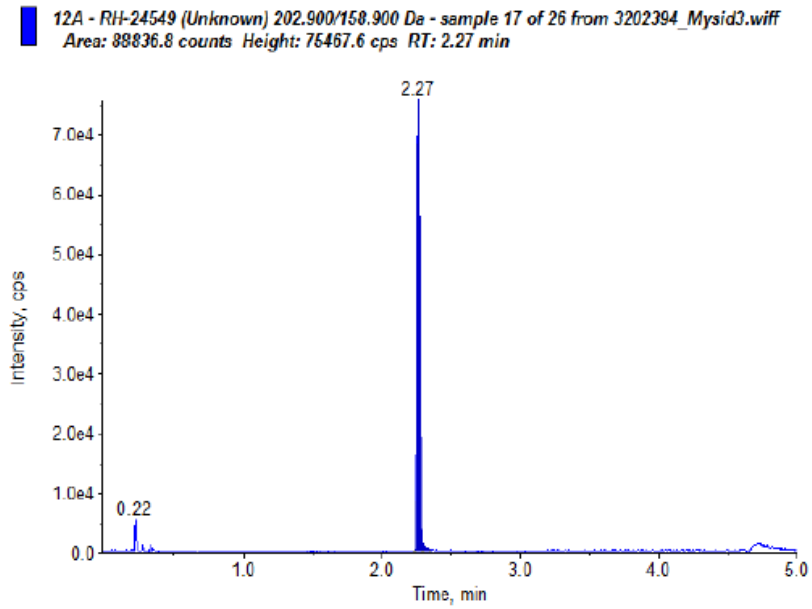


Figure A 14: **Chromatogram of RH-24549**

Conclusion

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

(Hugill E. 2020)

Method for Zoxamide in support of a *Lemna Gibba* study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The method has been acceptably validated for determination of the concentrations of the (RS)-zoxamide in aquatic medium with LOQ of 0.168 µg/L.*

Reference:	KCP 5.1.2 (f)/16
Report	Juckeland, D., 2020: Effects of zoxamide technical on *Lemna gibba* in a growth inhibition test under semi-static test conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 18 48 ALE 0005, GLP, Not published
Guideline(s):	SANCO/3029/99 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

A reverse phase-high performance liquid chromatography (RP-HPLC) method with mass spectrometric detection has been validated according to SANCO/3029/99 (2000) for the determination of zoxamide and its enantiomer's ratio in media of a *Lemna* toxicity test (BioChem project No. 18 48 ALE 0005). The method was based on Deierling & Hermann (2014), developed for the determination of zoxamide isomers in the formulated product, and adapted to the available equipment.

An external calibration (0.055 µg/L – 26.48 µg/L) with the reference item zoxamide was performed. The calibration panned from less than 65 % of the lowest validation concentration to approximately 120 % of the highest validation concentration.

The method was validated with fresh *Lemna* medium spiked with test item (5 replicates per fortification level) at concentrations of approximately 50% of the lowest test item concentration (0.168 µg/L) and approximately 120 % of the highest concentration (292.9 µg/L).

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

Equipment

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

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

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

Results and discussions

The method was validated with fresh *Lemna* medium spiked with test item (5 replicates per fortification level) at concentrations of approximately 50% of the lowest test item concentration (0.168 µg/L) and approximately 120 % of the highest concentration (292.9 µg/L).

Table A 62: Summary of recovery experiments

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

Accuracy and precision / repeatability

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

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

No zoxamide was detected in the control solutions.

Specificity

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

Linearity

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

Limit of quantification

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

Limit of detection

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

Matrix effects

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

Storage stability

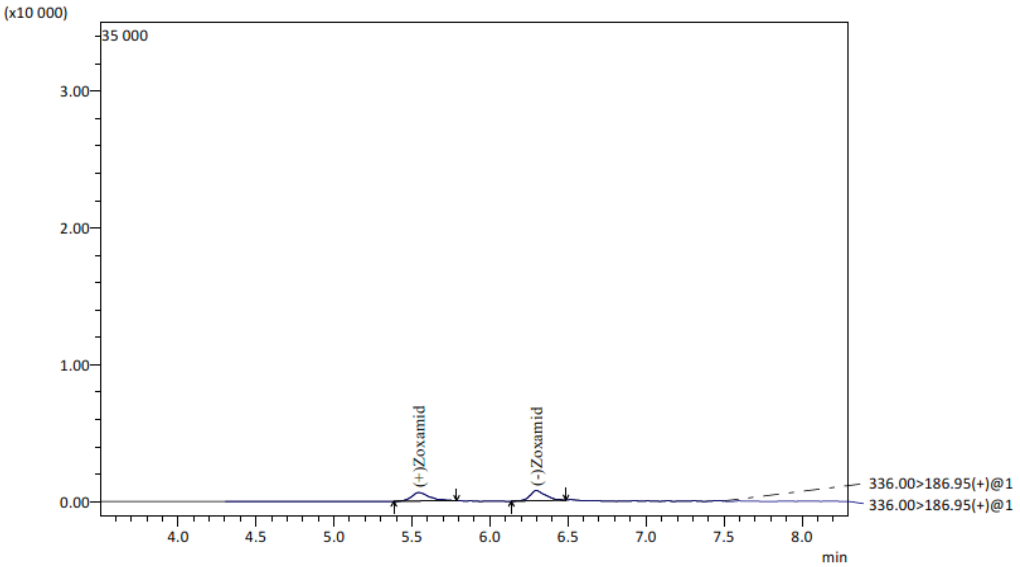
Stability was not investigated. Moreover, there were no sample extracts or solvent standards that were stored for > 24 hours in the refridgerator (at about 4°C). The maximum storage time of deep-frozen test samples (at ≤ -18°C) was 27 day (< 30 days).

Table A 63: Characteristics of the analytical method for the determination of zoxamide in aquatic medium

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

The following figures show typical chromatograms.

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



Quantitative Results

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

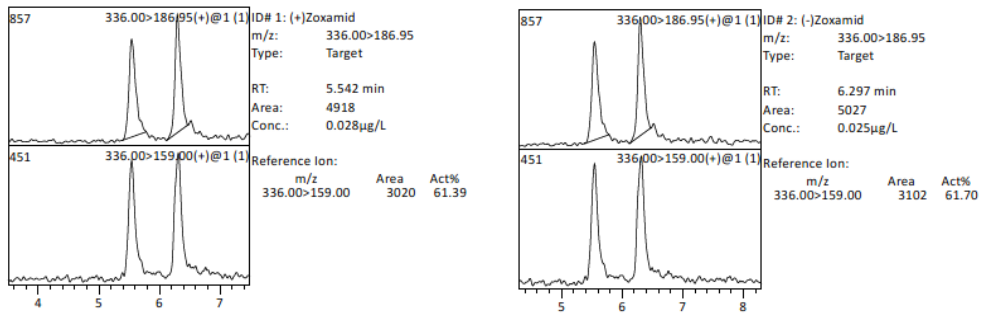
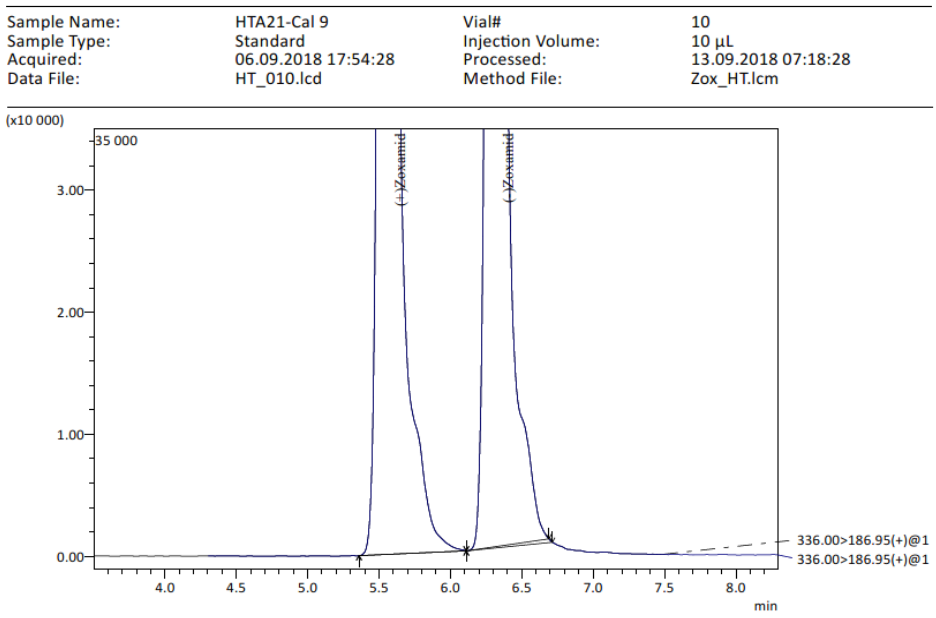


Figure A 15: Chromatogram of the lowest calibration standard



Quantitative Results

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

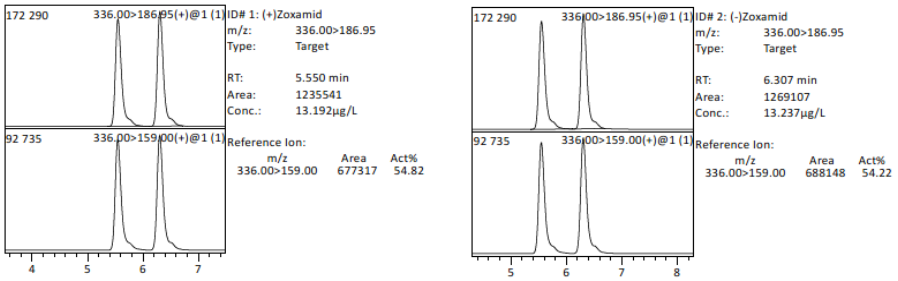


Figure A 16: Chromatogram of the highest calibration standard

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

Table A 64: Enantiomeric ratios

Samples	Mean enantiomeric ratio
Calibration	1.04
Validation low	1.05
Validation high	1.05
Treatment 9	1.05
Treatment 8	1.00
Treatment 7	1.01
Treatment 6	1.06
Treatment 5	1.00
Treatment 4	1.07
Treatment 3	1.07

Conclusion

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

The enantiomeric ratio of the test item is slightly different from 1 (1.00 to 1.07) but close enough to 1 to state that zoxamide existed as the racemate in the specimens.

(Juckeland D. 2020)

Method for RH-117,281

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *High-performance liquid chromatography (HPLC) combined with UV detection method has been acceptably validated in amended study (Milligan et al., 2020). The LOQ of method is set 0.015 mg a.i./L as the lowest fortification concentration for which a mean recovery of 70-110% was obtained.*

Reference:	KCP 5.1.2 (f)/17
Report	Milligan, Amanda L., Martin, Kathy H., Schneider, Suzanne Z., 2020: Final report addendum for RH-117,281 technical: An early life-stage toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) Gowan Crop Protection Ltd., UK Eurofins EAG Agroscience, LLC, USA, Report No. 129A-143A, GLP, Not published
Guideline(s):	OCSPP 850.1400 (2016) SANCO 3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/18
Report	Drottar, Kurt R., Krueger, Henry O., 1998: RH-117,281 Technical: An Early Life-Stage Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*) Rohm & Haas Company, USA, Report no. 97RC-0078 Wildlife International Ltd., USA, Amendment No. 129A-143A GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4

Deviations:	Two sets of day 0 analytical samples were collected. The first set of samples contained an interference in the chromatography. An additional set of samples was collected and analysed using a direct injection method which removed the interference. The protocol states that method validation which bracelets the concentrations of the study will be conducted and approved by Dr. Sandra Ferris prior to initiation of the definitive. The method was actually validated after test initiation since the first set of samples contained an interference in the chromatography. A new analytical method was developed concurrently with the Day 0 samples. However, formal validation of the method occurred after test initiation. The deviations did not adversely affect the results and the integrity of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Zoxamide (RH-117,281) in saltwater samples of a fish early life-stage (ELS) study with sheepshead minnow (*Cyprinodon variegatus*) was analysed with a high-performance liquid chromatography (HPLC) combined with UV detection.

The method comprised a direct analysis of the aqueous samples, diluted with filtered saltwater as needed, and analysed by reverse-phase chromatography with UV detection at an LOQ of 0.01 mg a.s./L. Test item concentrations were calculated using linear regression from external matrix matched (filtered saltwater) standard solutions.

The method was validated with filtered saltwater spiked with the test item zoxamide at four concentrations of 0.005, 0.015, 0.100 and 0.400 mg/L. In addition, saltwater samples fortified at 0.015, 0.100 and 0.400 mg a.s./L were analysed concurrently with the test item samples to observe procedural recoveries during the test item measurements.

In an amendment to this study (Milligan et al., 2020), the analytical method used to analyse test solution samples in the original study were assessed for compliance with current SANCO guidance. The specificity of the method (confirmatory method) and its repeatability, as well as the freezer storage stability of samples and the stability of sample extracts (in case storage of samples was required) were assessed based on review of the original raw data.

Equipment

Instrument: Hewlett-Packard Model 1090 High Performance Liquid Chromatograph with diode array detector (DAD) or a Waters 486 Tunable Absorbance Detector

Column: Phenomenex Inertsil ODS-2 (250 nun x 4.6 nun, 5, µm particle size)

Mobile phase: Solvent A: Water
Solvent B: Acetonitrile

Gradient:

Time (min)	%A	%B	Curve
0.01	70	30	--
1.00	70	30	Hold
10.0	0	100	Linear
12.0	0	100	Hold
12.1	70	30	Step
17.0	70	30	Hold

Flow rate: 1.00 mL/min
Injection volume: 200 µL
Stop time: 17 minutes
Oven temperature: 40°C

Retention time: Zoxamide (RH-117,281): 13 minutes

Results and discussions

Table A 65: Summary of recovery experiments from the original study report

Matrix	Fortification level (mg/L)	Recovery	Mean recovery	Acceptable recovery	Acceptable RSD
Saltwater (fish medium)	0.005	107, 108, 108 %	108 %	70 – 110 %	20 %
	0.015	103, 104, 104 %	104 %		
	0.100	99.5, 99.8, 99.2 %	99.5 %		
	0.400	99.1, 100, 99.4	99.5 %		

During the study, matrix samples were fortified with RH-117,281 technical at 0.015, 0.100 and 0.400 mg a.s./L to determine recovery and to evaluate method performance. Summary information of the additional data is given in the following table.

Table A 66: Summary of recovery experiments from the amendment to the study report

Sample		Concentration RH-117,281 (mg a.s./L)		Percent Recovery[2]	Mean measured[5] (mg a.s./L)	Mean % Recovery ($\bar{x}$) Std. Dev. (SD) RSD[5]
Number (129A-143A-)	Type	Fortified	Measured[12]			
MAB-2	Matrix Blank	0.0	< LOQ	-	-	-
MAB-3	Matrix Blank	0.0	< LOQ	-		
MAB-4	Matrix Blank	0.0	< LOQ	-		
MAB-5	Matrix Blank	0.0	< LOQ	-		
MAB-6	Matrix Blank	0.0	< LOQ	-		
MAB-7	Matrix Blank	0.0	< LOQ	-		
MAS-4	Matrix Fortification	0.015	0.0155	103	0.0149	($\bar{x}$) = 99.4
MAS-7	Matrix Fortification	0.015	0.0169[3]	113[3]		SD = 8.95
MAS-10	Matrix Fortification	0.015	0.0142	94.8		RSD =
MAS-13	Matrix Fortification	0.015	0.0079[3]	53.2[4]		9.00%
MAS-16	Matrix Fortification	0.015	0.0144	96.1		
MAS-17	Matrix Fortification	0.015	0.0135	89.9		
MAS-5	Matrix Fortification	0.100	0.101	101	0.102	($\bar{x}$) = 102
MAS-8	Matrix Fortification	0.100	0.106	106		SD = 2.14
MAS-11	Matrix Fortification	0.100	0.100	100		RSD =
						2.10%
MAS-14	Matrix Fortification	0.100	0.103	103		
MAS-17	Matrix Fortification	0.100	0.102	102		
MAS-20	Matrix Fortification	0.100	0.101	101		
MAS-6	Matrix Fortification	0.400	0.398	99.6	0.394	($\bar{x}$) = 98.4
MAS-9	Matrix Fortification	0.400	0.374	93.6		SD = 2.53
MAS-12	Matrix Fortification	0.400	0.400	100		RSD =
						2.57%
MAS-15	Matrix Fortification	0.400	0.390	97.5		
MAS-18	Matrix Fortification	0.400	0.401	100		
MAS-21	Matrix Fortification	0.400	0.399	99.7		
			Mean[3] =	100		

		Standard Deviation[5] =	5.13		
		RSD[5] =	5.13%		

1 During the study the method limit of quantitation (LOQ) was set at 0.0100 mg a.s./L and was calculated as the product of the lowest standard (0.0100 mg a.s./L) and the dilution factor of the matrix blank (1.00). The SANCO method limit of quantitation (LOQ) was defined as 0.015 mg a.s./L, the lowest matrix fortification concentration in which a mean recovery of 70-110% was obtained.
2 Results were generated using Excel 4.0 V. Manual calculations may differ slightly.
3 The measured value was extrapolated below the curve.
4 The low recovery was likely due to a fortification error. This result has been excluded from the calculation of the mean as an outlier.
5 Results were generated using Microsoft Excel 2010. Manual calculations may differ slightly.

Accuracy and precision / repeatability

The method is considered acceptable according to SANCO/3029/99 rev. 4 for accuracy with mean recovery values in the range of 70 to 110% for each fortification level and for precision with relative standard deviations of ≤ 20% per level.

A mean recovery 99.4% and %RSD = 9.00 has been calculated for 5 replicates fortified at 0.015 mg a.s./L, a mean recovery 102% and %RSD = 2.10 has been calculated for 5 replicates fortified at 0.1 mg a.s./L, a mean recovery 98.4% and %RSD = 2.57 has been calculated for 5 replicates fortified at 0.4 mg a.s./L.

The precision of the method has been reported as the relative standard deviation (RSD) of repeatability at each fortification level and the overall RSD. In general, the RSD should be ≤ 20% per fortification level. The precision and repeatability criteria were met at the 0.015, 0.100 and 0.400 mg a.s./L matrix fortification levels.

Linearity

Calibration with matrix matched standard solutions (5 points) for zoxamide, with concentrations ranging from 0.010 to 0.200 mg a.s./L, were linear (r = 1.0). A representative calibration curve is presented in the study report.

Based on the amendment to the report, the calibration standard series were injected at the beginning and end of the analytical run with, in addition, a minimum of one standard injected following every five samples. One or more calibration curves were derived from regression analysis of the instrumental responses of the standards as an assessment of linearity. The linear calibration range exceeded the expected nominal concentrations in final dilutions/extracts by at least ±20% for samples with test concentrations of 0.075, 0.15 and 0.30 mg a.i/L. This was not achieved for study samples 0.019, 0.038 and the low matrix fortification 0.015 mg a.s./L (LOQ during the study).

Limit of quantification

During the study the method limit of quantitation (LOQ) was set at 0.0100 mg a.s./L and was calculated as the product of the lowest standard (0.0100 mg a.s./L) and the dilution factor of the matrix blank (1.00). The SANCO/3029/99 rev. 4 method limit of quantitation (LOQ) is defined as 0.015 mg a.s./L, the lowest matrix fortification concentration for which a mean recovery of 70-110% was obtained.

Matrix effects

Matrix matched standards were used.

Specificity

No interferences > 30 % LOQ were observed at or above the LOQ at the retention time of interest for the blank matrix solutions (filtered salt water solutions), demonstrating the specificity of the method.

Table A 67: Characteristics of the analytical method for the determination of zoxamide in fish medium

	Zoxamide
Specificity	The HPLC-DAD method is regarded specific with a typical UV spectrum and a typical R_f value of the analyte. Typical chromatograms are provided. Blank value < 30 % LOQ
Calibration (type, number of data points)Example equation:	5 points calibration with matrix matched standard solutions. Correlation coefficient r > 0.99. A representative calibration curve and calibration line equation is presented in the study report. Example equation: y = 1.366x + 1524.889, r = 1.000
Calibration range	0.0100 to 0.200 mg a.s./L (new: 0.015-0.200 mg a.s./L); linear
Assessment of matrix effects is presented	No (matrix matched standards used).
Limit of quantification (LOQ)	LOQ: 0.01 mg a.s./L (new: 0.015 mg a.s./L)

The following figures show typical chromatograms.

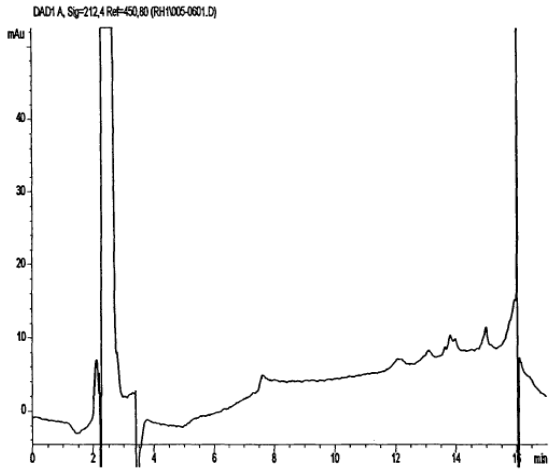


Figure A 17: A representative chromatogram of matrix blank

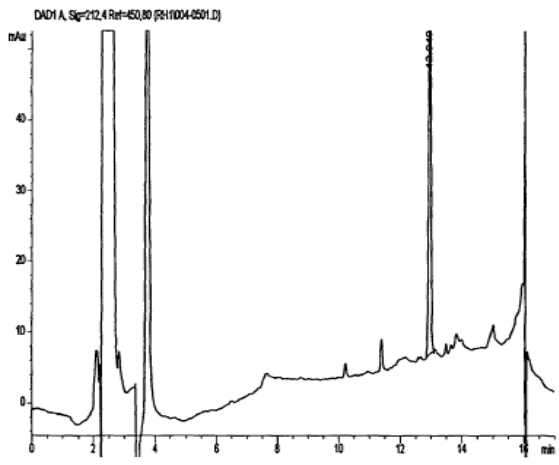


Figure A 18: A representative chromatogram of a 0.200 mg a.s./L RH-117,281 standard (40 ng on column)

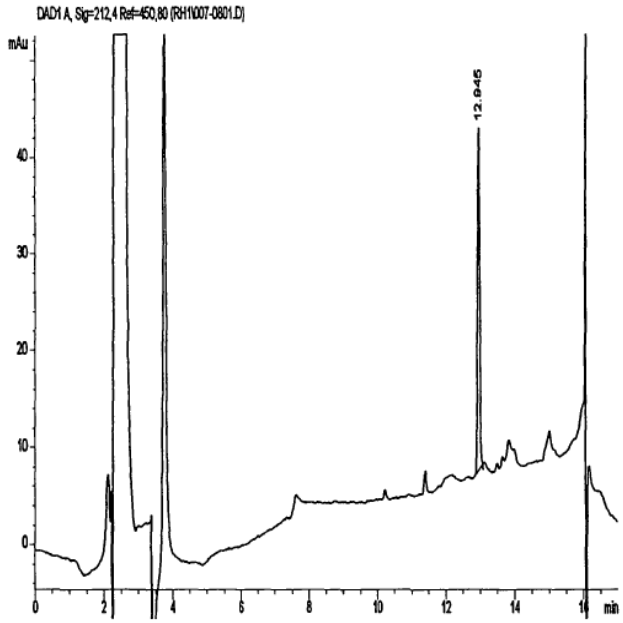


Figure A 19: A representative chromatogram of matrix fortification (0.100 mg a.s./L RH-117,281)

Conclusion

The analytical method for the determination of zoxamide in fish medium is regarded valid for the date of the time when the study has been performed. A re-evaluation according to current guidelines (SANCO/3029) confirmed this: The analytical findings met all data requirements with the exception of a confirmatory method.

(Drottar Kurt R., Krueger Henry O. 1998)

(Milligan A., Martin K., Schneider S. 2020)

Method for Zoxamide in support of bumble bee study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The high-performance liquid chromatography (HPLC) with UV-detection is fit for the determination of zoxamide (sum of both isomers) in test item stock solutions. The method was validated for the test item stock solutions of oral test in a 50% (w/v) sucrose solution and the contact test 0.5% (v/v) Triton X solutions. The method for the determination of zoxamide (racemate) in the test solutions of the bumble bee study has been acceptably validated according to SANCO/3029/99 rev. 4. and complies with SANTE/2020/12830, Rev.1. The limit of quantification (LOQ) is 50 g/L of zoxamide (sum) for contact test and 5 g/L for specimens for oral test.*

Reference:	KCP 5.1.2 (f)/19
Report	Amsel, K., 2018: Acute toxicity of Zoxium 240 SC to the bumblebee *Bombus terrestris* L. under laboratory conditions Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 17 48 BBA 0017, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of the active ingredient zoxamide (sum of isomers and isomers ratio) in the test solutions has been analytically verified. The determination has been conducted by an in-house developed method using high performance liquid chromatography (HPLC) with DAD-detection. During the course of this study, the analytical method has been validated according to SANCO/3029/99 rev. 4 using external standard calibration.

The method was validated by spiking the two matrices from the biological part; the validations were done separately for both the solution of the oral test (50% (w/v) sucrose solution) and the contact test (0.5% (v/v) Triton X solutions).

For the determination of zoxamide (sum), the concentrations of both isomers (sum of areas under the curves) were determined. The results were compared to the 5-point calibration data with external standard. For the determination of the zoxamide isomer ratio, the detector signals were registered and integrated using the data system. The sum of both peak areas in one chromatogram was set to 100% and the per-centage area of each peak was calculated.

The specimens, stored in a freezer at ≤ -18°C, were thawed at room temperature for 60-70 minutes, homogenised by shaking and diluted with 50% (v/v) water/methanol before analysis.

Equipment

HPLC System:	Shimadzu LC-20 HPLC system with a diode-array detector
Column:	Phenomenex-Lux, Cellulose-3, 150*2 mm, 3 µm

Mobile phase:	A: water with 5 mM ammonium carbonate pH9 B: acetonitrile with 5% (v/v) water
Gradient:	0.00 min → 50% A and 50% B 8.00 min → Stop
Flow rate:	0.5 mL/min
Column temp.:	25°C
Injection volume:	10 µL
Retention time:	3.7 min for *R*-zoxamide 5.3 – 5.4 min for *S*-zoxamide

Results and discussions

Table A 68: Summary of the validation results

Validation	No. of repli-cates	Nominal conc. of a.s. [mg/L]	Nominal conc. of a.s. regarding DF [mg/L]	Mean measured conc. of a.s. [mg/L]	Recalibra-tion factor (Rcf)	Dilution factor (DF)	Mean analysed conc. of a.s. [mg/L]	Mean recovery [% of nominal]	RSD [%]
contact test (matrix: 0.5% (v/v) TritonX solution)									
low conc.	5	50025	10.01	9.899	0.998	5000	49385	99	0.3
high conc.	5	106097	21.22	20.05	0.996	5000	99847	94	0.6
oral test (matrix: 50% (w/v) sucrose solution)									
low conc.	5	4970	9.939	10.19	1.001	500	5099	103	0.4
high conc.	5	10642	21.28	21.03	1.003	500	10543	99	0.2

Accuracy and repeatability/precision

Accuracy was tested by spiking sample matrix with test item at 2 concentrations levels. As a result, mean recoveries for each level were in the range 70-110%.

Repeatability is regarded acceptable for 5 replicates per concentration level with an RSD (relative standard deviation) of < 20% per level.

Linearity

Linearity was tested for 5 points at a concentration range of at least ± 20% of a.s. in the analytical solution, with correlation coefficient of > 0.99.

Limit of quantification

The limit of quantification (LOQ) was defined as the lowest successfully validated fortification level, i.e. 50 g/L zoxamide for specimens in the contact test matrix and 5 g/L zoxamide for specimens in the oral test matrix.

Matrix effects

Not relevant since matrix matched standard solutions were used.

Specificity

UV spectra from 200 to 300 nm were continuously recorded by the diode-array detector. Spectra of the peaks were compared to those of the reference item. Similar spectra with approximately equal absorption maxima, a constant chromatographic retention time and no interfering peaks were observed.

No interfering peaks were detected. Validation blank samples had peak areas of less than 30% of the lowest validated test item concentration.

Stability of sample extracts

The maximum storage time of deep-frozen stock solutions was < 30 days. Extracts for analysis were stored for < 24 hours in the refrigerator.

Table A 69: Characteristics of the analytical method for the validation of zoxamide in oral and contact bumblebee test solutions

	Zoxamide (sum of isomers)
Specificity	HPLC with UV-detection, similar spectra from 200 to 300 nm, constant retention time, no interfering peaks
Calibration (type, number of data points)	5-point calibration with external standard The calibration was linear, no weighting was used. Calibration curve equation: $y = 148181\ x - 872.688$, $r^2 = 0.9999943$
Calibration range	5.910 to 29.551 mg/L of zoxamide (sum)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected in both matrices
Limit of determination/quantification	LOD: 5.910 mg/L for contact test and oral test LOQ: 50 g/L (according to 10 mg/L, regarding DF) for contact test 5 g/L (according to 10 mg/L, regarding DF) for oral test

DF = dilution factor

The following figure shows atypical chromatogram.

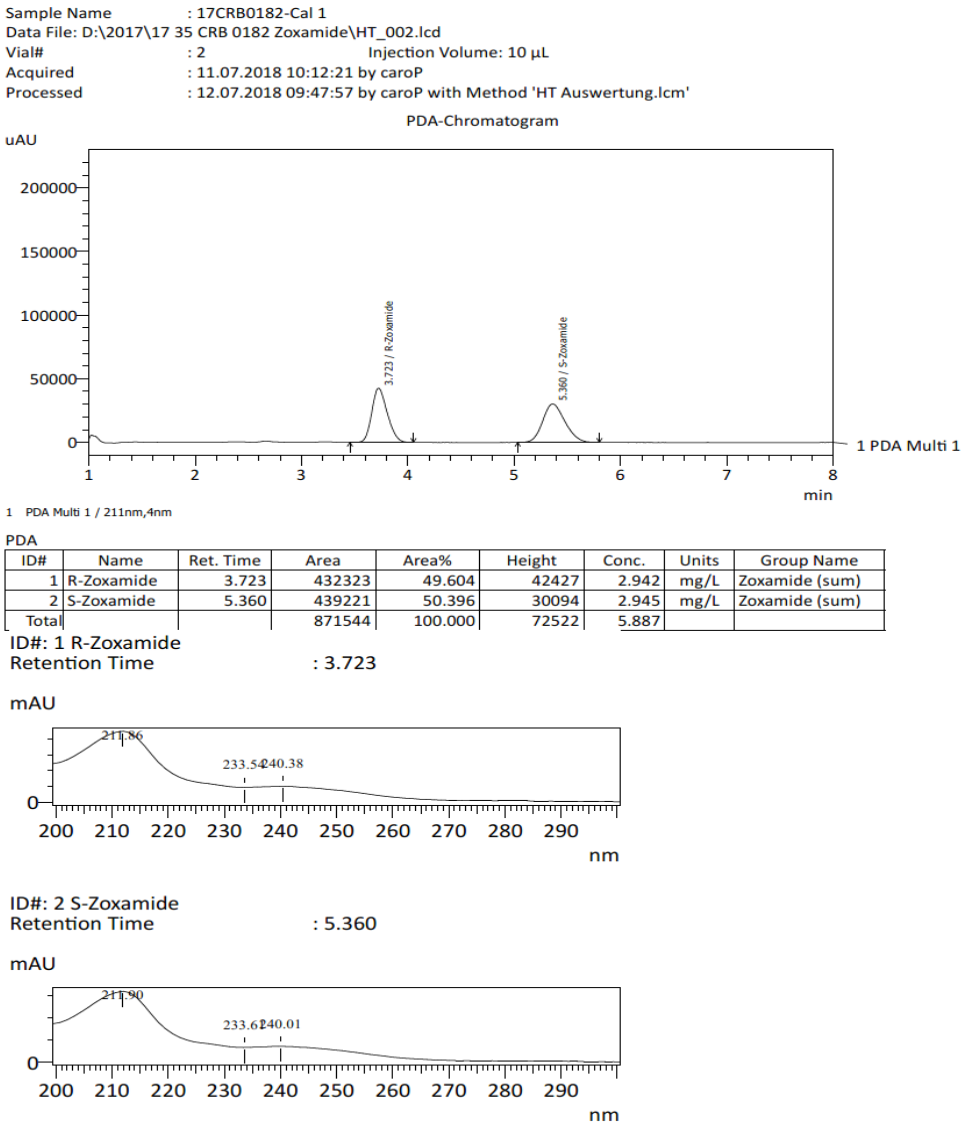


Figure A 20: Chromatogram of the lowest calibration standard of zoxamide (racemate)

As a result, the test item concentration in the oral toxicity test solution was analytically confirmed (100% of the nominal zoxamide concentration), as well as the test item concentration in the contact toxicity test solution (98% of the nominal zoxamide concentration). No active substance was detected in the control samples. The ratio of the R- and S-isomer of zoxamide in the test item solutions was 1:1.

Conclusion

An analytical method for the determination of the active ingredient zoxamide (racemate) in the test solutions of the bumble bee study was successfully validated and regarded acceptable according to SANCO/3029/99 rev. 4. The determination was conducted by high performance liquid chromatography (HPLC) with UV-detection. As a result, the nominal zoxamide concentrations were analytically verified. In addition, the 1:1 ratio of R- and S-Zoxamide in the test item stock solutions was confirmed.

The method is capable for the determination of zoxamide in the test solutions of an acute oral (50% (w/v) sucrose solution) and topical (0.5% (v/v) TritonX solution) bumble bee study, it fulfils all criteria of the guidance document SANCO/3029/99.

(Amsel K. 2018)

Method for Zoxamide in support of honey bee study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) method was acceptably validated in royal jelly diet with zoxamide at concentrations of 0.500 and 5000 µg a.i./g . Recoveries were averaged in the range 105 ± 2.36% with a limit of quantification (LOQ) of 0.500 µg a.i. at the lowest fortification level and the detection limit (MDL) of 0.250 µg a.i./g.*

Reference:	KCP 5.1.2 (f)/20
Report	Picard, Ch. R., 2018: Zoxamide: Honey bee (*Apis mellifera* L.) larval toxicity, repeated exposure Exigent LLC, A Gowan Group Company, USA Smithers Viscient, USA, Report No. 12791.6307, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

An analytical method for the determination of zoxamide present in royal jelly diet of honey bees has been validated according to SANCO/3029 rev. 4 (2000). Recovery samples were initially diluted with 80/20 acetone/purified reagent water (v/v) and were subsequently diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). Recovery samples were analysed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Equipment:

LC-MS/MS System:	MDS Sciex API 5000 mass spectrometer
Column:	Phenomenex Kinetex C18, 2.6 µm, 2.1 × 50 mm
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile

Gradient:

Time [min]	Solvent A [%]	Solvent B [%]
0.50	95.0	5.00
2.50	0.00	100
3.90	0.00	100
4.00	95.0	5.00
5.00	95.0	5.00

Flow rate:	0.600 mL/min
Column temp.:	40°C
Injection volume:	100 µL
Run Time	5.00 minutes

Retention time:	Approx. 2.7 min
Ionization Mode:	Positive (+) ESI
Scan type:	MRM
Q1/Q3 Masses:	336.094/187.20 amu

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

Results and discussions

Table A 70: Summary of recovery experiments

Sample ID: 12791-6308-	Sample Type	Fortified Concentration (µg a.s./g)	Retention Time (minutes)	Dilution Factor	Analytical Result (µg a.s./g)	Percent of Fortified
14	Reagent Blank	0.00	NA[a]	5000	<0.250[b]	NA
15	Control	0.00	NA	5000	<0.250	NA
16	Control	0.00	NA	5000	<0.250	NA
17	LOQ	0.500	2.73	5000	0.542	108
18	LOQ	0.500	2.74	5000	0.532	106
19	LOQ	0.500	2.74	5000	0.515	103
20	LOQ	0.500	2.74	5000	0.513	103
21	LOQ	0.500	2.74	5000	0.514	103
		Mean:	**2.74**		**0.523**	**105**
		SD[c]:	**0.00447**		**0.0131**	**2.63**
		% RSD[d]:	**0.163**		**2.51**	**2.51**
22	High	5000	2.74	25.000.000	5200	104
23	High	5000	2.74	25.000.000	5130	103
24	High	5000	2.73	25.000.000	5440	109
25	High	5000	2.73	25.000.000	5240	105
26	High	5000	2.74	25.000.000	5120	102
		Mean:	**2.74**		**5230**	**105**
		SD:	**0.00548**		**130**	**2.60**
		% RSD:	**0.200**		**2.49**	**2.49**
		Overall Mean:	**2.74**		**Overall Mean:**	**105**
		Overall SD:	**0.00483**		**Overall SD:**	**2.47**
		% RSD:	**0.176**		**% RSD:**	**2.36**
					N[e]:	**10**

[a] NA = Not Applicable
[b] Concentrations expressed as less than values were below the method detection limit (MDL). The MDL is dependent upon the lowest concentration calibration standard and the dilution factor of the controls (i.e., 0.0500 µg a.s./L × 5000 mL/g = 0.250 µg a.s./g).
[c] SD = Standard Deviation
[d] RSD = Relative Standard Deviation
[e] N = Total number of samples used to determine the overall mean, standard deviation (SD), and coefficient of variation (RSD)
NOTE: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Accuracy and precision / repaeatability

The accuracy and repeatability/precision of the procedure were determined at five different concentration levels.

Recoveries ranged from LOQ (0.500 µg/g) with 105 ± 2.63% (RSD: 2.51%) to the highest successfully validated dose (5000 µg/g) with 105 ± 2.60% (RSD: 2.49%).

The results for accuracy and precision showed RSD values < 10 %.

Recovery values were within the required range of 70-110 % (i.e. 105 ± 2.36%).

Repeatability with 5 replicates for each level showed RSD values < 20% per level.

Linearity

Linearity was checked by a 6-points calibration curve (single injections) using matrix matched standard solutions at a range of 0.500 and 5000 µg a.s./g ($r^2 > 0.99$).

Limit of quantification

The limit of quantification was 0.500 µg a.s./g, the lowest fortification level.

Limit of detection

The method detection limit (MDL) was 0.250 µg a.s./g.

Matrix effects

Matrix-matched standard solutions were used.

Specificity:

The LC-MS/MS method was regarded specific for the analyte. No significant interference was observed at the retention time of zoxamide in blank standard samples (i.e. < 30% of the analyte peak area).

Storage stability

The concentration of zoxamide in refrigerated royal jelly diet remained stable for at least four days without degradation of the active substance, which allows the use of the same treated diets for three days.

Table A 71: Characteristics of the analytical method for the determination of zoxamide in royal gelee

	Zoxamide
Specificity	HPLC-MS/MS method. Mass spectra provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	6-point calibration with external matrix-matched standard. Linear. Calibration curve equation: y = 155262.7229 x – 249.6153, r^2 = 0.999
Calibration range	0.500 and 5000 µg a.s./g
Assessment of matrix effects is presented	No. Matrix-matched standard solutions were used.
Limit of determination/quantification	LOQ: 0.500 µg a.s./g. LOD: 0.250 µg a.s./g

The following figure shows typical chromatogram.

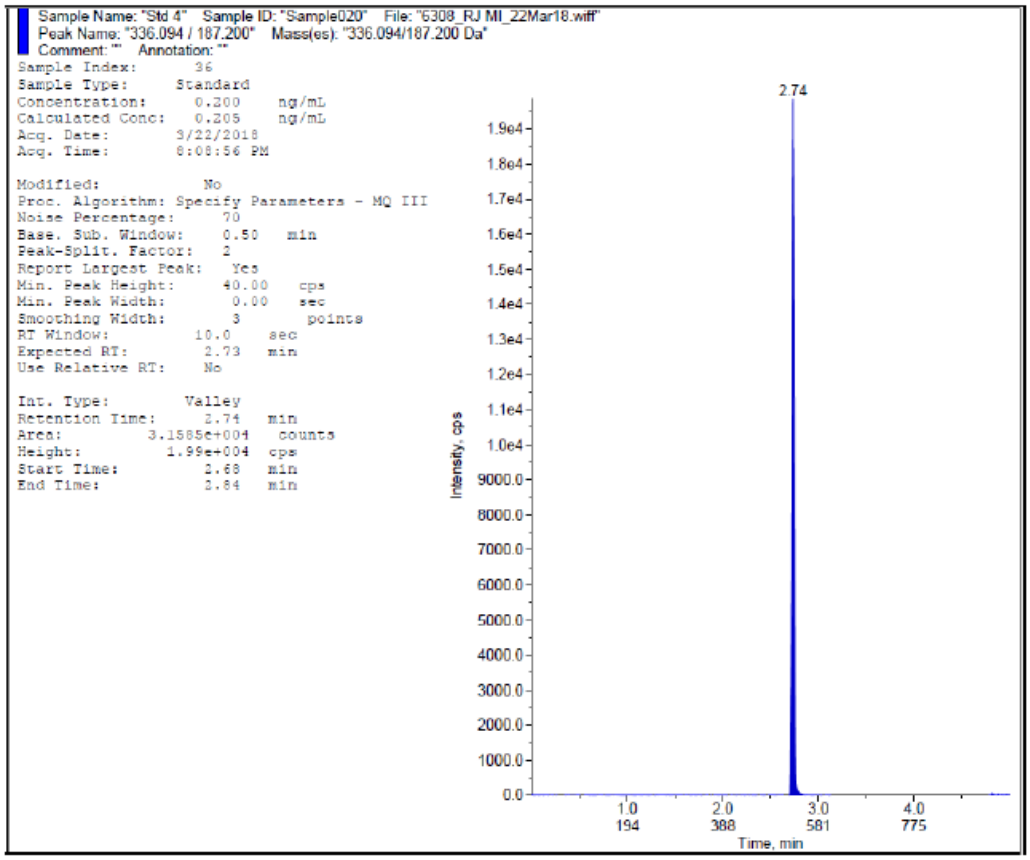


Figure A 21: Chromatogram of a calibration standard

Conclusion

The method for the determination of zoxamide in royal gelee diet was regarded valid according to SANCO/3029/99 rev. 4 (2000).

(Picard Ch. R. 2018)

Method for Zoxamide in support of an earthworm study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *A highly specific HPLC-MS/MS method has been acceptably adapted to the expected concentration range of this study. The zoxamide was analysed in soil by a method Jooß (2013), using extraction with acetonitrile and separation by reverse-phase high-pressure liquid chromatography (HPLC) and tandem mass spectroscopic (MS/MS) determination of zoxamide with matrix-matched external standards. The LOQ was defined in the context of the study as the lowest fortification level, i.e. 0.051 mg/kg zoxamide in wet soil specimens, equivalent to 0.063 mg/kg zoxamide in dry weight soil.*

Reference:	KCP 5.1.2 (f)/21
Report	Friedrich, S., 2020: Effects of Zoxium 240 SC on the reproduction of the earthworm *Eisenia andrei* in artificial soil 5% peat Gowan Crop Protection Ltd., UK BioChem agrar, Germany, Report No. 17 48 TEC 0009, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

A highly specific HPLC-MS/MS method for the determination of zoxamide in soil has been validated according to SANCO/3029/99 rev. 4.

The active substance zoxamide was analysed in soil specimens by a method developed by Jooß (2013), using extraction with acetonitrile and separation by reverse-phase high-pressure liquid chromatography (HPLC) with tandem mass spectroscopic (MS/MS). Matrix-matched external standards were used. The method of Jooß (2013) has been adapted to the expected concentration range of this study.

5 g (± 0.05 g) soil sample were weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of the acetonitrile phase were transferred to autosampler vials and diluted.

The analytes were determined after extraction with two mass transitions (zoxamide: m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

Instrument:	Aglient 1200/64100 system with a triple quadrupole mass spectrometric detector
Column:	ACE Excel 3µm C18-AR 100*2.1 mm

Mobile phase: A: Water containing 1 mL/L formic acid and 5 mmol/L ammonium formate
B: Methanol containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00		Stop

Flow rate: 0.4 mL/min
Run time: 7.00 min (3.0 min post-run equilibration)
Ionisation: ESI (electrospray ionisation) positive
Ion mode Zoxamide:
m/z 336 → m/z 159 (quantifier ion)
m/z 336 → m/z 187 (qualifier ion)

Results and discussions

Table A 72: Summary of recovery experiments

Validation	Replicates	Nominal conc. (mg/kg dry weight)	Nominal conc. (mg/kg moist soil)	Main analysed conc. (mg/kg)	Mean recovery (%)	RSD (%)
Low	5	0.063	0.051	0.050	99	3.8
Medium	5	0.632	0.506	0.509	101	2.2
High	5	29.09	23.28	23.82	102	2.7

Accuracy and precision / repeatabiltiy

The results for accuracy and precision showed RSD values < 10 % for each spiking level and overall. Recoveries were in the expected range of 70-110%.
Untreated samples showed residues < LOD.
Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was demonstrated for matrix-matched calibration curves (8 concentration levels) of zoxamide in the range of 0.80 – 84 µg/L (corresponding to 0.05 – 5.25 mg /kg zoxamide in dry soil). This covers ranges from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with correlation coefficients r^2 all greater than 0.99, demonstrating satisfactory linearity.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 0.051 mg/kg zoxamide in wet soil specimens, equivalent to 0.063 mg/kg zoxamide in dry weight soil.

Matrix effects

Matrix effects were compensated by matrix-matched calibration standards.

Specificity

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

Storage stability

Sample extracts and solvent standards were stored for max. two days in the refrigerator or the cooled autosampler (at 4-8°C). A stability of 8 days for zoxamide in sample extracts and for solvent standard solutions was demonstrated in BioChem study no. 18 35 CRX 0023 by Thomas (2020).
Frozen soil samples (≤-18°C) were stored during the course of this study for at maximum 425 days. Storage stability of zoxamide in deep frozen soil samples has been demonstrated for a period of 633 days in BioChem study no. 18 35 CRX 0023 by Thomas (2020).

Table A 73: Characteristics of the analytical method validation for the determination of zoxamide in artificial soil

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ) Mass sprectrum provided.
Calibration (type, number of data points)	8-point calibration with matrix-matched standard. The calibration was linear. Calibration curve equation: $y = 192.895 x + 34.631$, $r^2 = 0.99944$
Calibration range	0.80 – 84 µg/L in analytical samples (corresponding to 0.05 – 5.25 mg /kg zoxamide in dry soil)
Assessment of matrix effects is presented	No (matrix-matched standards).
Limit of quantification	0.051 mg/kg zoxamide in moist soil (as received), equivalent to 0.063 mg/kg zoxamide in dry soil and 1.0 µg./L in the analytical sample.

The following figure shows a representative chromatogram.

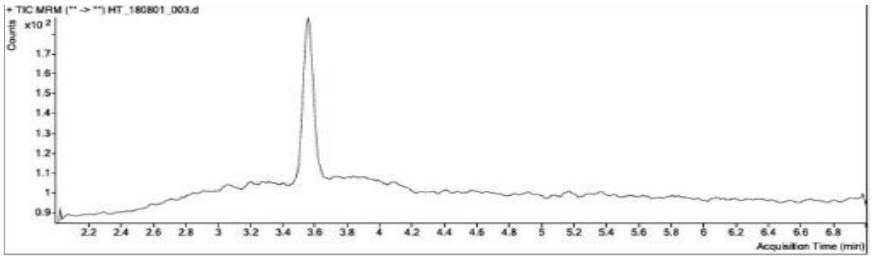


Figure A 22: Chromatogram of the lowest zoxamide standard

Conclusion

A highly specific HPLC-MS/MS method for the determination of zoxamide in soil has been validated according to SANCO/3029/99 rev. 4.

(Friedrich S. 2020)

Method for RH-127450 in support of an earthworm study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU,*

	GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021: *The analytical procedure by LC-TOF/MS for the determination of RH-127450 in soil has been validated according to SANTE/2020/12830, Rev.1 guideline.* *The LOQ of the procedure is 0.016 mg/kg for RH-127450.*

Reference:	KCP 5.1.2 (f)/22
Report	Gray, J., 2021: RH-127450: Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202376, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	On one occasion the minimum temperature recorded was 17.8°C, below the guideline minimum of 18°C, but within rounding. The number of juveniles in replicates E-H inclusive of the solvent control was not assessed until day 57 as the assessment took longer than expected due to the high numbers of juveniles recorded. This did not affect the number of juveniles present as the adults had been removed at day 28. At day 56 the soil moisture content was equivalent to > 60% MWHC (the guideline maximum, not referenced in the protocol) at 0.53, 0.95, 1.72 and 5.56 mg a.s./kg dry substrate, with a maximum of 61.42% at 1.72 mg a.s./kg dry substrate. However, as all the validity criteria were met, these deviations were not considered to have had any impact on the integrity or outcome of the study. For the application rates where the soil moisture content was equivalent to >60% MWHC at day 56 the mean number of juvenile worms produced at each rate was within the overall range for the study (minimum of 408 in the water control and maximum of 517 at 10 mg a.s./kg dry substrate). As the NOEC for reproduction was determined to be 10 mg a.s./kg dry substrate and the EC_{10}, EC_{20} and EC_{50} values were >10 mg a.s./kg dry substrate the deviations in % MWHC were not considered to have any impact on the number of juveniles produced at the affected application rates or on the integrity or outcome of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

An analytical method SMV 3202376-03V for the determination of RH-127450 in artificial soil (containing 10 % peat) has been validated according to SANCO 3029/99 rev. 4 (2000).

5.0 g soil was dispensed into 50 mL Falcon tubes. Samples were fortified as required and shaken well by hand to mix. 20 mL of MeCN was added and the sample extracted by shaking on a rotary shaker, at a set speed of 200 rpm and for a set period of 10 minutes. This was then sonicated for a set period of five minutes before being centrifuged at a set speed of 2500 rpm for a set period of 15 minutes. A portion of the supernatant was transferred to a suitable vial for LC/MS analysis. If required samples were diluted with unfortified control extract to bring the response within the calibration range. Aliquots of the samples were injected onto a liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) system.

Equipment for LC-TOF/MS Analysis

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

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

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

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 5 µL
Retention time: 2.28 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 302.07 → m/z 186.9500-186.9900

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

Results and discussions

Table A 74: Summary of recovery experiments

10% peat soil	0.016 µg/mL	0.5 µg/mL	15 µg/mL	Overall
Mean Recovery (%)	99.0	96.9	94.7	96.9
CV 0(%)	3.72	1.22	0.785	2.87

Accuracy and precision / repeatability

The accuracy and repeatability / precision of the procedure was determined by fortifying 5 g aliquots of 10% peat soil medium (in quintuplet) at three different concentrations. The results showed RSD values of < 10 % for each spiking level and the soil. Recovery values were within the required range of 70-110 %. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by 7-points calibration curve (single injections) using matrix matched standards at a range of 0.0005 – 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.016 mg/kg.

Matrix effects

Matrix-matched standards were used for calibration.

Specificity

The LC-TOF/MS analysis method was considered specific to RH-127450 as the analysis of untreated 10% peat soil samples exhibited no interferences exceeding 30% of the proposed LOQ of 0.016 mg/kg at the retention time of RH-127450.

Storage stability

Frozen samples were stored for a maximum of three days and were analysed on the day they were removed from storage. However, freezer storage stability of RH-127450 residues in 10% peat soil was confirmed for at minimum 7 days.

Calibration standard solutions were prepared on the day of use.

Table A 75: Characteristics of the analytical method for the determination of RH-127450 in artificial soil

	RH-127450
Specificity	The LC-TOF/MS method is considered specific. Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Matrix-matched standard calibration. 7 points calibration; linear with 1/x weighting. Correlation coefficient r >0.99. Calibration data and calibration line equation presented in the study report. y = 6.46473e6 x + 1201.55291, r = 0.99842
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No. Matrix-matched standard calibration.
Limit of quantification (LOQ)	LOQ: 0.016 mg/kg

The following figure shows a typical chromatogram.

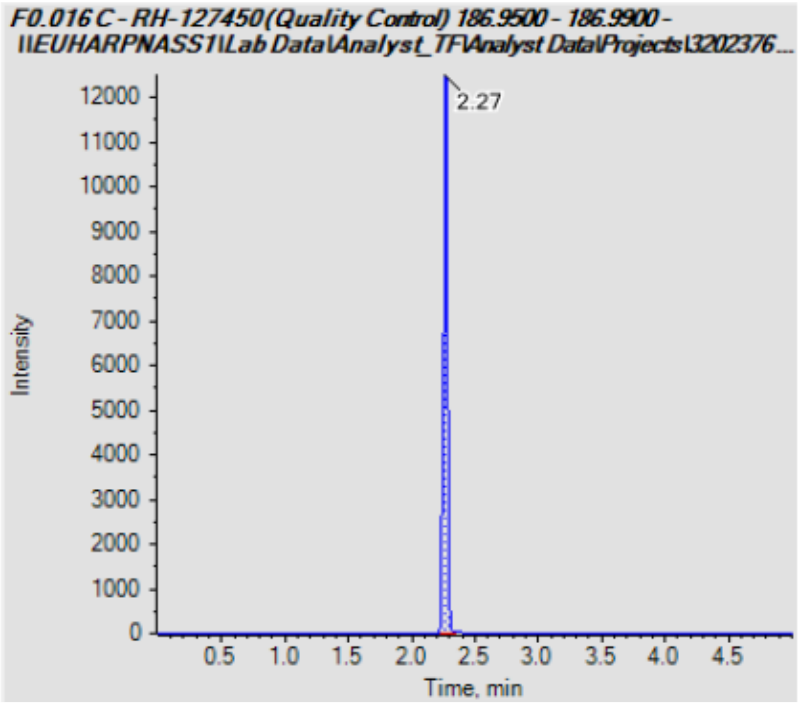


Figure A 23: **Chromatogram - 10% peat soil fortified with RH-127450 at 0.016 mg/kg**

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4 (2000).

(Gray J. 2021)

Method for RH-24549 in support of an earthworm study

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) system or liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system for the determination of RH-24549 in artificial soil acceptably validated according to* *Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes (SANTE/2020/12830, Rev.1, 24. February 2021). The LOQ is 0.016 mg/kg for RH-24549.*

Reference:	KCP 5.1.2 (f)/23
Report	Gray, J., 2021: RH-24549 - Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202395, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	The light intensity deviated from the guideline times of 16:8 hours light:dark on one occasion during the reproduction phase of the test, when the timer was still recording a dark period after 15.05 hours. The soil used in the study had a peat content of 8.1% and not 10% as stated in the protocol. The pipette used to adjust the soil moisture content on day 35 (06 August), day 42 (13 August 2019) and day 49 (20 August 2019) was found to have failed to meet the required criteria when calibrated on 27 August 2019. At Day 56 the soil moisture content was equivalent to >60% MWHC (the guideline maximum, not referenced in the protocol) in the water control and at 0.16, 0.29, 0.53, and 1.72 mg a.s./kg dry substrate, with a maximum of 62.80% at 0.53 mg a.s./kg dry substrate. Chemical analysis at Day 0 – recovery in one of the eight treatment rates was below the 80% minimum requirement of the analytical procedure documentation (actual recovery for the 5.56 mg a.s./kg dry substrate was 73.05% of nominal, equivalent to 4.06 mg a.s./kg). As there was 100.14% recovery at the maximum rate of application and the applied dose of 4.06 mg a.s./kg dry substrate was higher than for the 3.09 mg a.s./kg dry substrate a dose sequence was maintained. These deviations were not considered to have had an adverse impact on the study as all the validity criteria were met. For the application rates where the soil moisture content was equivalent to >60% MWHC at Day 56 the mean number of juvenile worms produced at each rate was within the overall range for the study (minimum of 131 at 10 mg a.s./kg dry substrate and maximum of 185 at 5.56 mg a.s./kg dry substrate). As the NOEC for reproduction was determined to be 10 mg a.s./kg dry substrate and the EC50 value was >10 mg a.s./kg dry substrate the deviations in %MWHC were not considered to have any impact on the number of juveniles produced. In addition, the EC10 and EC20 were >1.72 mg a.s./kg dry substrate the maximum rate at which the

	%MWHC exceeded the guideline maximum. Therefore, the increase in %MWHC is not considered to have had an adverse impact on the integrity or outcome of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

An analytical method (SMV 3202395-01V, and later revisions) was used to confirm the concentrations of RH-24549 in artificial soil samples (10% peat). The method was validated according to SANCO 3029/99 rev. 4.

5.0 g soil was dispensed into 50 mL Falcon tubes. Samples were fortified as required and shaken well by hand to mix. 20 mL of extraction solvent (1% formic acid in acetonitrile) was added and the sample extracted by shaking on a rotary shaker, at a set speed of 200 rpm for a set period of 10 minutes. This was then sonicated for a set period of five minutes and centrifuged at 2500 rpm for 15 minutes. A portion of the supernatant was transferred to a suitable vial for LC/MS analysis. Where required, the sample was diluted with unfortified control extract to bring the response within the calibration range. Samples were analysed by injection via liquid chromatography with time-of-flight mass spectrometry (LC-TOF/MS) or liquid chromatography triple quadrupole mass spectrometry (LC-TQMS) system.

Equipment for LC-TOF/MS analysis

Instrument: AB Sciex TripleTOF5600 + coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software

Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm

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

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

Flow rate: 0.50 mL/min

Column temp.: 50°C

Injection volume: 10 µL

Retention time: 2.16 min approx.

Analysis time: 5 min

Ionisation: Electrospray (ESI), negative

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

Equipment for LC-TQMS analysis

Instrument: API 5000 TQMS coupled to Shimadzu SIL-30ACMP Quaternary HPLC

Column: Waters BEH Phenyl, 1.7 µm, 50 x 2.1mm

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

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

Flow rate: 0.50 mL/min
Column temp.: 50°C
Injection volume: 3 µL
Retention time: 2.28 min approx.
Analysis time: 5 min
Ionisation: Electrospray (ESI), positive
Ion mode: m/z 202.9 → m/z 158.9

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

Results and discussions

Table A 76: Summary of recovery experiments

10% peat soil	0.016 mg/kg	0.5 g/kg	10 mg/kg	Overall
	LC-TOF/MS			
Mean Recovery (%)	87.9	86.5	89.2	87.9
CV (%)	1.99	3.02	1.63	2.46
	LC-TQMS			
Mean Recovery (%)	98.8	99.8	96.6	98.4
CV (%)	2.5	3.7	4.4	3.6

Accuracy and precision / repeatability

The accuracy and precision of the procedure were determined by fortifying 5 g aliquots of 10% peat soil medium in quintuplet at three different concentrations. The results showed RSD values of < 10 % for each spiking level and the soil. Recovery values were within the required range of 70-110 %. Untreated samples showed residues < LOQ. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by an 8-points calibration curve (single injections) over a concentration range of 0.0005 – 0.1 µg/mL using matrix-matched standard solutions. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.016 mg/kg.

Matrix effects

Matrix-matched standards were used for calibration.

Specificity

Both methods were considered specific to RH-24549 as the analysis of untreated 10% peat soil samples exhibited no interferences exceeding 30% of the proposed LOQ of 0.016 mg/kg for RH-24549 at the retention time of RH-24549.

Storage stability

Frozen samples were stored for a maximum of six days. However, freezer storage stability of samples was assessed and confirmed for at minimum 6 days. Calibration standard solutions were prepared on the day of use.

Table A 77: Characteristics of the analytical method validation for the determination of RH-24549 in artificial soil

	RH-24549
Specificity	LC-TOF/MS and LC-TQMS are regarded as highly specific. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix-matched standard calibration. 8-points calibration; linear with 1/x weighting; r >0.99. Calibration data and calibration line equation presented in the study report. y = 2.16e + 006 x +-189, r = 0.9993
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix matched standards).
Limit of determination/quantification	LOQ: 0.016 mg/kg

The following figur shows a typical chromatogram.

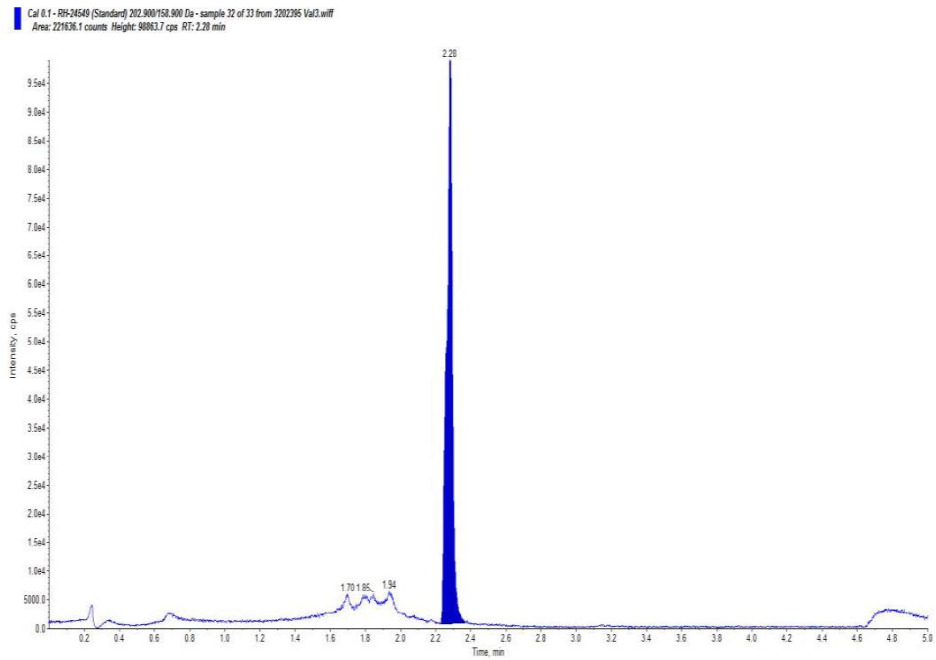


Figure A 24: Representative chromatogram of a calibration standard containing 0.1 µg/mL RH-24549 in unfortified control extract using LC-TQMS

Conclusion

The analytical procedure has been successfully validated in terms of specificity, linearity, precision, accuracy and LOQ in accordance with guideline requirements of SANCO/3029/99 rev. 4 (2000).

(Gray J. 2021)

Methods for metabolite RH-163353 in soil

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The method has been validated for the analysis of RH-163353 in 10% peat soil. A mean overall recovery in the range 80-120% and a coefficient of variation of ≤ 20% what is considered acceptable. The LOQ of the procedure is 0.016 mg/kg for RH-163353.*

Reference:	KCP 5.1.2 (f)/24
Report	Gray, J., 2021: RH-163353: Effect on reproduction in the earthworm *Eisenia fetida* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202389, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	At day 28 the soil moisture content was equivalent to 37.59% MWHC (below the guideline minimum of 40%, not referenced in the protocol) at 10 mg a.s./kg dry substrate. Any moisture loss was made up when the soil was returned to the test vessel. The data shows that the Day 56 the moisture content was >40% MWHC and there was no effect on survival (all LCx values >10 mg a.s./kg dry substrate) or the percentage gain in adult weight over the 28-day exposure period. In addition, the number of juveniles produced was greater than in both controls with all the ECx values being >10 mg a.s./kg dry substrate. Therefore, this deviation was considered to have no impact on the integrity or outcome of the study.
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/25
Report	Gray, J., 2021: RH-163353: Collembolan reproduction test in soil – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202390, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	Each test vessel was weighed and the weight adjusted to the day 0 level by addition of RO water on day 14. In this way individual vessel moisture loss was made up to

	the correct level whereas the use of a single moisture control vessel as stated in the protocol does not account for the variation in loss between vessels. The process was repeated at Day 21 to ensure optimum environmental conditions were maintained as far as possible throughout the exposure period. The light period was less than 16 hours on one occasion over the 28-day exposure period. The mean % MWHC was below the minimum of 40% stated in the protocol for all treatments and the control throughout the study. As the % MWHC to obtain the correct soil structure had been determined to be 40%MWHC, the lowest level specified, any moisture lost during the mixing process would have resulted in the final % MWHC being below the selected level. These deviations are considered to have no impact on the study integrity. In addition, the OECD 232 guideline states 'The moisture content of the testing soil should be optimised to obtain a loose porous structure allowing the collembolans to enter into the pores. This is usually between 40-60 % maximum WHC.' Therefore, the % MWHC that provides the correct soil structure may be outside the 40-60 % range. It is considered that as the validity criterion of ≥100 juveniles per replicate was achieved in the controls and at all rates of application the lower % MWHC has had no impact on the reproductive output.
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/26
Report	Gray, J. 2021: RH-163353: Effect on reproduction of *Hypoaspis* (*Geolaelaps*) *aculeifer* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202391, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	At day 14 the mean % MWHC was below the minimum of 40 % stated in the protocol for the water control and in the 0.82, 1.47, 2.65 and 4.76 mg a.s./kg dry substrate treatments. Moisture content over the exposure period also varied by >10% in both controls and at 0.82, 1.47, 2.65 and 4.75 mg a.s./kg dry substrate. This deviation was not considered to have had an adverse impact on the conduct or outcome of the study as the validity criteria were met in both water an solvent controls. In addition, there was no effect on the parental generation (NOEC > 50 mg a.s./kg dry substrate and all LCx values > 50 mg a.s./kg dry substrate). An adverse effect on reproduction was only recorded at 50 mg a.s./kg dry substrate where the moisture content was maintained at > 40 % MWHC throughout the exposure period.
GLP:	Yes
Acceptability:	Yes

Materials and methods

Similar analytical procedures SMV 3202389-01V and SMV 3202390-01V and following versions were used to determine the concentration of the analyte RH-163353 in samples of the test media (artificial soil with 5 or 10 % peat) from earthworm and *Folsomia*/*Hypoaspis* studies, respectively. The procedures have been validated according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-163353 were determined by extracting soil samples with an extraction solvent (1% formic acid in acetonitrile/acetone 3:1 v/v), then diluting further with unfortified control sample extract to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) system:

1. 2 - 5.0 (±0.05) g soil (depending on the residue level) was dispensed into 50 mL Falcon tubes.
2. Samples were fortified as required and shaken well by hand to mix.
3. 20 mL extraction solvent was added.
4. Samples were extracted for a set period of 10 minutes on a rotary shaker set to 200 rpm, sonicated for a set period of five minutes then centrifuged at a set speed of 2500 rpm for 10-15 minutes.
5. If required, samples were filtered through 0.2 µm PTFE syringe filter and/or diluted with unfortified control extract, and a portion of each extract was transferred to a suitable vial for LC/MS analysis.
6. Aliquots of the samples were injected onto the 5600 TOF-MS system.

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

Equipment for LC-TOF/MS analysis

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

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

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	10 µL
Retention time:	2.16 min approx.
Analysis time:	5 min
Ionisation:	Electrospray (ESI), positive
Ion mode:	m/z 332.04 → m/z 186.939-186.989

Equipment for enantiomeric ratio analysis

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

For more detailed information on the MS conditions, please refer to the study report.

Results and discussions

Table A 78: Summary of recovery experiments – earthworm study

10% peat soil	0.016 mg/kg	0.1 g/kg	15 mg/kg	Overall
Mean Recovery (%)	82.5	81.4	83.3	82.4
CV (%)	3.7	2.5	8.0	5.0

Table A 79: Summary of recovery experiments – *Hypoaspis* and *Folsomia* study

10% peat soil	0.16 mg/kg	1.0 g/kg	50 mg/kg	Overall
Mean Recovery (%)	75.8	84.6	82.4	80.9
CV (%)	2.1	2.8	1.0	5.2

Accuracy and precision / repeatability

The accuracy and repeatability/precision of the procedure was determined by fortifying artificial soil medium in quintuplet at three different concentrations. The results for accuracy and precision showed RSD values < 10 % for each spiking level and overall. Recoveries were in the expected range of 70-110%. Untreated samples showed residues < LOD. Mean overall recovery values in the range 80-120% and a coefficient of variation of ≤ 20% were considered acceptable to demonstrate repeatability.

Linearity

Linearity was checked by at least 7-points calibration curve (single injections) over the concentration range 0.0005 – 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ was set to 0.016 mg/kg.

Matrix effects

Matrix matched standards were used.

Specificity

The LC-TOF/MS analysis method was considered specific to RH-163353 as the analysis of untreated 10% peat soil samples exhibited no response exceeding 30% of the proposed LOQ of 0.016 mg/kg for RH-163353 from either the test substance or interferences at the retention times of RH-163353.

Storage stability

The maximum freezer storage period for samples was one day. However, freezer storage stability of residues in soil samples has been confirmed over a period of 7 days.

Sample extracts were analysed within 24 hours (stored refrigerated in the autosampler). Also, calibration standard solutions were prepared on the day of use.

Table A 80: Characteristics of the analytical method validation for the determination of RH-163353 in artificial soil

	RH-163353
Specificity	LC-TOF/MS is regarded specific. Mass spectrum is provided. Blank value < 30 % LOQ.
Calibration (type, number of data points)	Matrix-matched standard calibration. At least 7-points calibration curve; linear with 1/x weighting; r > 0.99. Calibration data and calibration line equations presented in the study reports. y = 9.88522e5 x + 111.6068, r = 0.99703 (earthworm) y = 7.2954e5 x + 41.99930, r = 0.99975 (collembolan, *Folsomia*)
Calibration range	0.0005 – 0.1 µg/mL
Assessment of matrix effects is presented	No. Matrix matched standards used.
Limit of quantification/determination	LOQ: 0.016 mg/kg

The following figure shows a typical chromatogram.

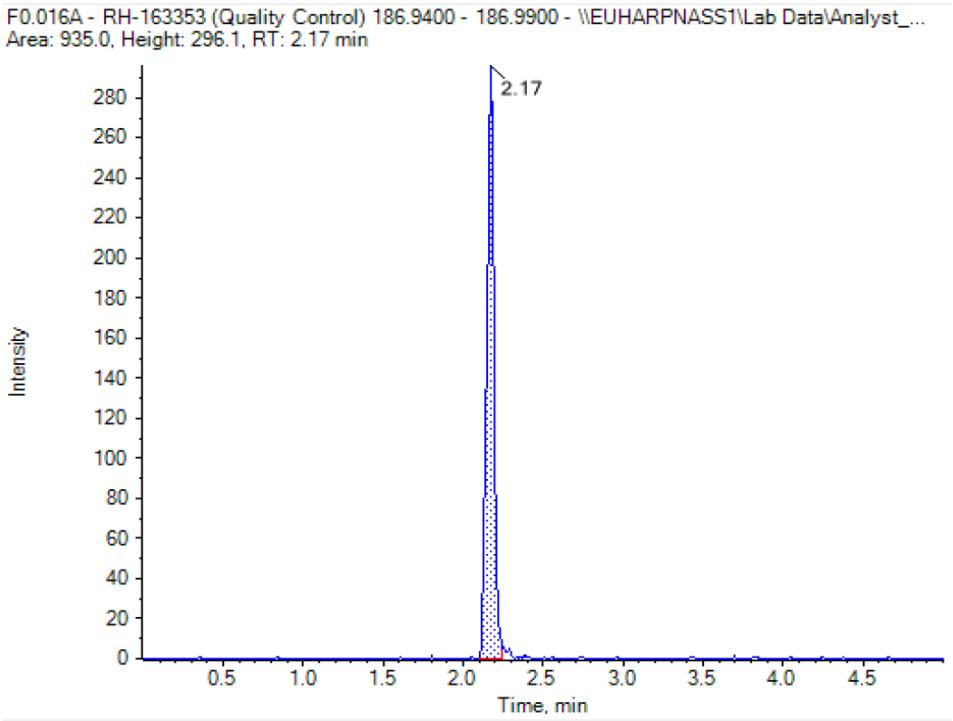


Figure A 25: Representative chromatogram of soil fortified with RH-163353 at 0.016 mg/kg

The mean enantiomeric ratio of RH-163353 on day 14 samples (*Hypoaspis* study) was 47.31% for Isomer A and 52.69% of Isomer B. The mean enantiomeric ratio of RH-163353 on day 28 samples (*Folsomia* study) was 47.93% Isomer A and 52.07% Isomer B in comparison to means of 48.91% and 51.09% of Isomers A and B respectively in the calibration samples. The enantiomeric ratio on the Certificate of Analysis is 48.6:51.4. The enantiomeric ratio observed in the samples did not differ from the Certificate of Analysis value by more than ±1.5%, and was therefore deemed to have not changed during the lifetime of the tests.

Conclusion

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

The enantiomeric ratio of RH-163353 was stable in the soils during the lifetime of the tests.

(Gray J. 2021 a, b, c)

Methods for Zoxamide in soil

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The HPLC-MS/MS method is fit for determination of zoxamide (sum of enantiomers) despite its shortcomings. The lowest calibration point was not determined at 30% of the LOQ in accordance with SANTE/2020/12830 rev. 1.*

Reference:	KCP 5.1.2 (f)/27
Report	Schulz, L., 2020: Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK, BioChem agrar, Germany, Report No. 18 48 FEW 0001, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/28
Report	Schulz, L., 2021: Effects of Zoxium 240 SC on earthworms under field conditions Gowan Crop Protection Ltd., UK, BioChem agrar, Germany, Report No. 19 48 FEW 0002, GLP, Not published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	No
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the analytical verification of the soil concentrations of Zoxamide after application of the test item Zoxium 240 SC (containing nominally 240 g/L zoxamide).

The determination of zoxamide (sum of enantiomers) was conducted by a HPLC-MS/MS method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 (Jooß S., 2013). It was adapted to the equipment available at BioChem agrar. Changes to the original method are reported and laboratory validation data according to SANCO/3029/99 were compiled prior to the test item measurements.

5 g soil were weighed (to ±0.05 g) into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 200 µL of the acetonitrile phase were pipetted into autosampler vials and diluted with 790 µL water and 10 µL formic acid. After extraction, the analyte was determined monitoring two mass transitions (m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

HPLC parameters for chiral separation of zoxamide stereoisomers:

Instrument:	Shimadzu HPLC system with a triple quadrupole mass spectrometric detector (Shimadzu LC-20ADXR system with LCMS-8040 mass spectrometric detector, auto-sampler and LabSolutions Version 5.91)
Column:	ACE Excel 3 µm C18-AR 100 * 2.1 mm

Mobile phase: A: water containing 1 mL/L formic acid and 5 mmol/L ammonium formate
B: acetonitrile containing 1 mL/L formic acid and 5 mmol/L ammonium formate

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI (electrospray ionisation) positive, MRM
Ion mode Zoxamide:
m/z 336 → m/z 187 (quantifier)
m/z 336 → m/z 159 (qualifier)

Results and discussions

Recovery findings

Table A 81: Validation results (study no. 18 48 FEW 0001)

Validation level	Replicates	Nominal concentration [mg/kg d.w.]	Mean analysed concentrations [mg/kg d.w.]	Mean recovery [%]	RSD [%]
Low	5	0.0563	0.0577	102	5.9
High	5	0.563	0.614	109	4.4

Table A 82: Validation results (study no. 19 48 FEW 0002)

Validation level	Replicates	Nominal concentration [mg/kg d.w.]	Mean analysed concentrations [mg/kg d.w.]	Mean recovery [%]	RSD [%]
Low	5	0.0117	0.0105	90	12.1
Medium	5	0.1173	0.1198	102	2.2
High	5	1.173	1.214	103	12.2

Accuracy and precision / repeatability

The accuracies, reported as mean recovery, and precision/repeatability, reported as relative standard deviation (RSD) are shown in the tables above. Mean recoveries for each level are in the range 70-110%, the RSD is < 20% per level. Blank values do not exceed 30% of the lowest validation concentration. The results fulfill the criteria of SANCO/3029/99 rev. 4.

Linearity

In study no. 18 48 FEW 0001, linearity was demonstrated for matrix-matched calibrations (6 concentration levels) in the range of 0.78 to 12.0 µg/L, with a correlation coefficient (r) greater than 0.99. This is equivalent to 0.044 - 0.68 mg/kg zoxamide in dry soil.

In study no. 19 48 FEW 0002, linearity was demonstrated for matrix-matched calibrations (8 concentration levels) in the range of 0.16 to 24.0 µg/L, with a correlation coefficient (r) greater than 0.99. This is equivalent to 0.009 to 1.41 mg/kg zoxamide in dry soil.

Limit of quantification

In study no. 18 48 FEW 0001, the limit of quantification (LOQ) was defined as the lowest successfully validated fortification level, i.e. 0.056 mg/kg zoxamide, per soil dry weight.

In study no. 19 48 FEW 0002, the limit of quantification (LOQ) was defined as the lowest successfully validated fortification level, i.e. 0.012 mg/kg zoxamide, per soil dry weight.

Limit of detection

The limit of detection was defined as 30% of the LOQ or 0.003 mg/kg dry weight in study no. 19 48 FEW 0002.

Matrix effects

Matrix-matched calibration was used to compensate possible matrix effects. Nevertheless, no significant matrix effect was found, comparing standards prepared in matrix and in solvent.

In study no. 19 48 FEW 0002, the slope of a solvent calibration differed by approximately 3% from the slope of a matrix-matched calibration (matrix effect -3%).

Specificity

The method is regarded as highly specific. It shows a characteristic retention time of the analyte and uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two mass transitions per analyte for quantification and confirmation, respectively. No interfering peaks above 30% of LOQ were detected.

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 606 and 639 days, respectively. The freezer storage stability of zoxamide residues in soil samples was confirmed for 643 days (21 months) in study 18 48 FEW 0001. For this, freshly spiked soil samples were analysed concurrently to the stored samples in the freezer.

Storage stability of sample extracts and solvent standards

Sample extracts (and calibration solutions) were stable for 8 days at 4-8°C, covering their maximum storage periods of 6 and 8 days.

Table A 83: Characteristics of the analytical method validation for the determination of zox-amide in field soils

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	6-point and 8-point calibration with external standard. Individual calibration data and curve equation are presented in the report. The calibration was linear over the whole calibration range. Example calibration curve equations: $y = 12540.6 x – 341.9$, $r^2 = 0.9996$ (study no. 18 48 FEW 0001) $y = 395686 x – 33270$, $r^2 = 0.9995$ (study no. 19 48 FEW 0002)
Calibration range	0.78 to 12.04 µg/L in soil extracts; equivalent to 0.044 - 0.684 mg/kg zoxamide in dry soil (study no. 18 48 FEW 0001) 0.16 to 24.0 µg/L in soil extracts; equivalent to 0.009 - 1.41 mg/kg zoxamide in dry soil (study no. 19 48 FEW 0002)
Assessment of matrix effects	Matrix-matched calibration was used to compensate possible matrix effects. Nevertheless, no significant matrix effect was found, comparing standards prepared in matrix and in solvent. In study no. 19 48 FEW 0002, the slope of a solvent calibration was differed by approximately 3% from the slope of a matrix-matched calibration (matrix effect -3%).
Limit of quantification (LOQ)	0.056 mg/kg zoxamide per soil dry weight (study no. 18 48 FEW 0001) 0.012 mg/kg zoxamide per soil dry weight (study no. 19 48 FEW 0002)

The following figure shows a representative chromatogram.

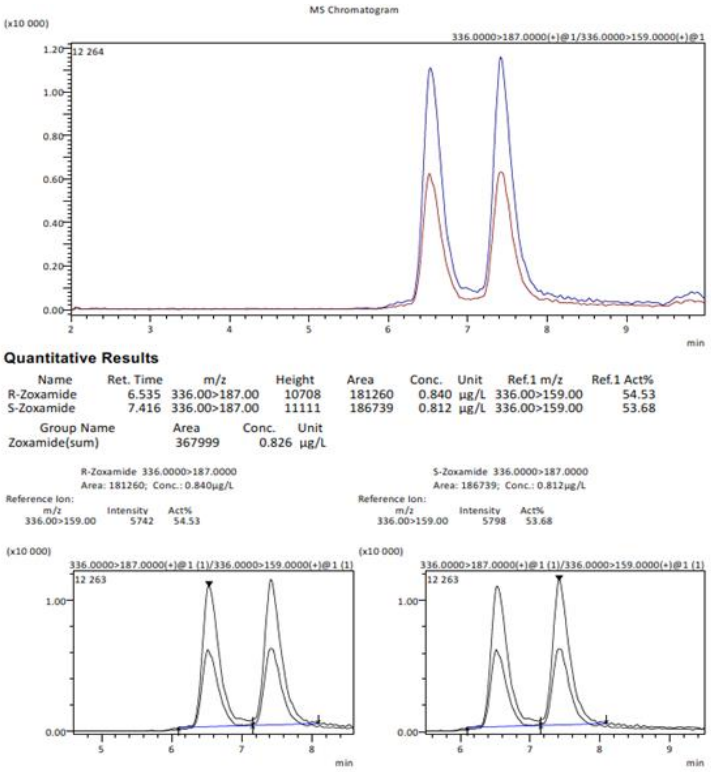


Figure A 26: Chromatogram of zoxamide (racemate) of the lowest matrix standard (study no. 19 48 FEW 0002)

Conclusion

The analytical method is applicable for the determination of zoxamide in soil. It fulfills the criteria of SANCO/3029/99 rev. 4, which was in place during the performance of the studies.

Nevertheless, the re-validation of the method is also in line with SANTE/2020/12830 rev. 1 dated February 2021 (with the slight restriction that the lowest calibration point was not determined at 30% of the LOQ).

(Schulz L. 2020)

(Schulz L. 2021)

Methods for metabolite RH-141455 in soil

These active substance related studies have already been provided to the RMS Latvia. Thus, the summary of the studies is only presented for completeness sake. The studies are only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The analysis by LC-FT/MS and LC –TOF/MS is fit for the determination of RH-141455 in artificial soil based on validation criteria. The LOQ of the procedure is 0.2 mg/kg. However, should be mentioned that the limit of quantification for residues in soil should not be more than 0.05 mg/kg according to Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purpose (SANTE/2020/12830, Rev.1, 24. February 2021).*

Reference: KCP 5.1.2 (f)/29

Report	Gray, J., 2021: RH-141455: Collembola reproduction test in soil – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202382, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	During the main test at day 28 the mean % MWHC was below the minimum of 40% stated in the protocol in both the controls and in the 1.47 and 4.76 mg a.s./kg dry substrate treatment rates. Moisture content over the exposure period varied by >10% in both controls and at 0.82, 1.47, 2.65 and 4.75 mg a.s./kg dry substrate. However, these deviations were not considered to have had an adverse impact on the conduct or outcome of the study as the validity criteria were met. In addition, the OECD 232 guideline states 'The moisture content of the testing soil should be optimised to obtain a loose porous structure allowing the collembolans to enter into the pores. This is usually between 40-60% maximum WHC.' Therefore, the % MWHC that provides the correct soil structure may be outside the 40 – 60 % range. It is considered that as the validity criterion of ≥ 100 juveniles per replicate was achieved in the controls and at all rates of application the lower % MWHC has had no impact on the reproductive output.
GLP:	Yes
Acceptability:	Yes

and

Reference:	KCP 5.1.2 (f)/30
Report	Gray, J., 2021: RH-141455: Effect on reproduction of *Hypoaspis* (Geolaelaps) *aculeifer* – Amended final report 1 Gowan Crop Protection Ltd., UK Smithers ERS Ltd., UK, Report No. 3202383, GLP, Not published
Guideline(s):	SANCO 3029/99 rev. 4 (2000)
Deviations:	The light period was less than the 16 hours specified. The analytical data confirmed that the applied dose was in excess of the required 50 mg a.s./kg but showed it was also outside the accepted range of 80-100% of nominal (+29.28% at Day 0 and + 50.01% at Day 14). However, as the dose was in excess of that required it is considered that this confirms no adverse effect on survival or reproduction at the intended dose. As all the validity criteria were met. Therefore, these deviations were not considered to have had any impact on the integrity or outcome of the study.
Acceptability:	Yes

Materials and methods

The same analytical procedure (SMV 3202383-01V, later updated to SMV 3202383-02V to add LC-TOF/MS data and minor corrections and to SMV 3202383-03V to add stability data) and SMV 3202383-04V was used to determine the concentration of the analyte RH-141455 in samples of the test medium (artificial soil) from *Folsomia* and *Hypoaspis* studies. The procedures have been validated according to SANCO 3029/99 rev. 4. The principles of the method and the results of the method validations are presented hereunder.

Concentrations of RH-141455 were determined by extracting soil samples with an extraction solvent, then diluting further with unfortified control sample extract to bring the response within the calibration range. Samples were analysed by injection onto a liquid chromatography LC-FT/MS or LC-TOF/MS system:

1. 2.0 (± 0.05) g soil was dispensed into 50 mL Falcon tubes.

2. Samples were fortified as required and shaken well by hand to mix.
3. 40 mL extraction solvent (acetonitrile/acetone 2:1, v/v containing 1% formic acid) was added.
4. Samples were extracted on a rotary shaker set to 200 rpm for a set period of 10 minutes, sonicated for a set period of five minutes then centrifuged at a set speed of 2500 rpm for 5 minutes.
5. An aliquot of the final extract was filtered through 0.2 µm PTFE syringe filter and diluted if required with filtered unfortified control extract. A portion of the extract was transferred to a suitable vial for LC/MS analysis.
6. Aliquots of the samples were injected onto the ThermoScientific Q-Exactive or the 5600 TOF-MS system.

Equipment for LC-FT/MS analysis

Instrument:	Thermo Scientific Q-Exactive coupled to Shimadzu SIL-30AC Quaternary HPLC system. Xcalibur 4.1SP2 data collection software
Column:	Scherzo SM-C18, 3µm, 50 x 2.0mm
Mobile phase:	A: 0.1% formic acid in water (LC-MS grade) B: 0.1% formic acid in acetonitrile (LC-MS grade)

Time [min]	A [%]	B [%]
0.01	95	5
3.00	0	90
3.90	0	90
4.00	95	5
5.00	95	5

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	50 µL
Retention time:	4.5 min approx.
Analysis time:	5 min
Ionisation:	Heated Electrospray (HESI), negative
Ion mode:	m/z 232.94 → m/z 188.953-189.003

Equipment for LC-TOF/MS analysis

Instrument:	AB Sciex Triple TOF 5600 + coupled to Shimadzu SIL-30ACMP Quaternary HPLC system. Analyst TF 1.7.1 data collection software
Column:	Scherzo SM-C18, 3.0 µm, 50x 2.0 mm
Mobile phase:	A: 0.1% formic acid in water (LC-MS grade) B: 0.1% formic acid in acetonitrile (LC-MS grade)

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

Flow rate:	0.50 mL/min
Column temp.:	50°C
Injection volume:	25 µL
Retention time:	3.8 min approx.

Analysis time:	5 min
Ionisation:	Electrospray (ESI), negative
Ion mode:	m/z 232.95 → m/z 188.953-189.003

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

Results and discussions

Table A 84: Summary of recovery experiments

Artificial soil	LC-FT/MS			
	0.2 mg/kg	**1.0 g/kg**	**75 mg/kg**	**Overall**
Mean Recovery (%)	106	102	94.6	101
CV (%)	3.80	3.58	2.33	5.64
	LC-TOF/MS			
Mean Recovery (%)	112	116	104	111
CV (%)	3.01	2.64	2.19	5.22

Accuracy and precision / repeatability

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

Linearity

Linearity was checked by 8-points calibration curve (single injections) using matrix matched standards at a range of 0.001 to 0.1 µg/mL using calibration solutions prepared in unfortified control extract. The correlation coefficient (r) was greater than 0.99. The equation of the calibration graph was linear with 1/x weighting.

Limit of quantification

The LOQ of the procedure is 0.2 mg/kg.

Matrix effects

Matrix matched standards were used.

Specificity

Both methods were considered specific to RH-141455 as the analysis of untreated artificial soil samples exhibited no interferences > 30% of the proposed LOQ from the matrix at the retention time of the analyte.

Storage stability

In study no. 3202383 frozen samples were stored for a maximum of one day and were analysed on the day they were removed from storage. In study no. 3202382 frozen samples were stored for a maximum of six days and were analysed on the day they were removed from storage. However, freezer storage stability of RH-141455 residues in soil samples has been assessed to be at minimum 29 days. Calibration standard solutions were prepared on the day of use.

Table A 85: Characteristics of the analytical method for the determination of RH-141455 residues in artificial soil

	RH-141455
Specificity	Both methods (LC-FT/MS and LC-TOF/MS) are considered specific. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix matched standard calibration. 8-points calibration curve (single injections); linear with 1/x weighting; r > 0.99. Calibration data and calibration line equation presented in the study report. y = 5.58972e6 x + 78.15356, r = 0.99978
Calibration range	0.001 – 0.1 µg/mL
Assessment of matrix effects is presented	No (matrix matched stanstards were used).
Limit of quantification/determination	LOQ: 0.2 mg/kg

The following figure shows a typical chromatogram.

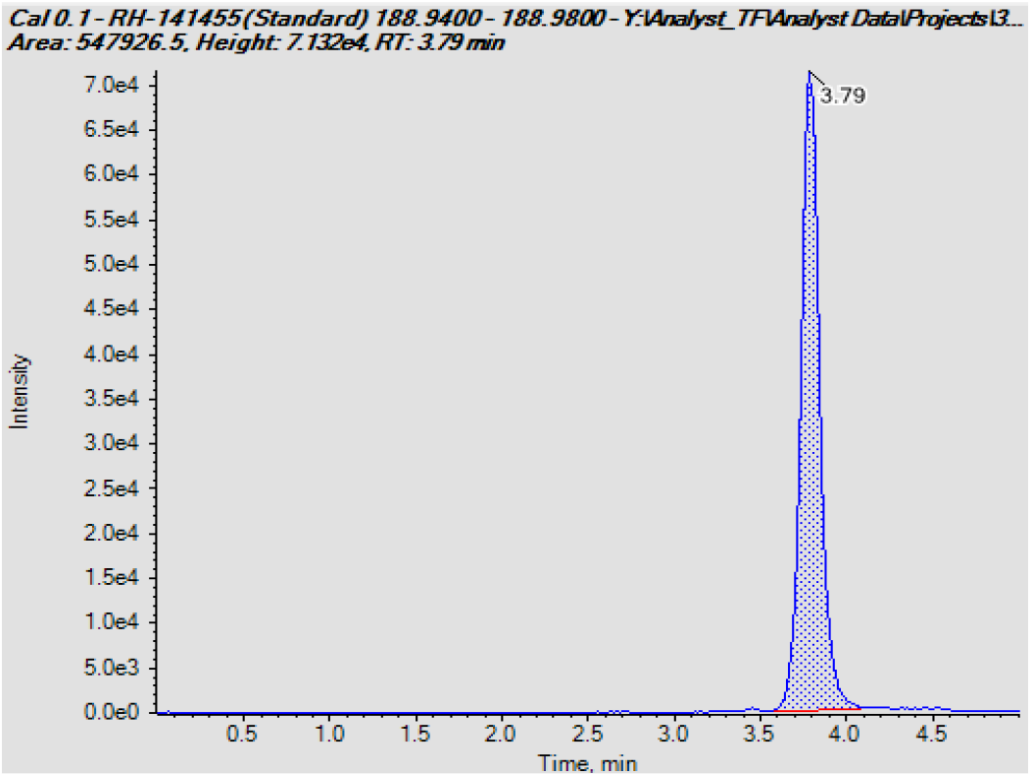


Figure A 27: Chromatogram of a of calibration standard containing 0.1 µg/mL RH-141455 in unfortified control extract using LC-TOF/MS

Conclusion

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

(Gray J. 2021)

Method for Zoxamide in artificial soil

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The used method is suitable for applied test item concentrations in soil specimens. The method for determination of zoxamide was analysed by validated method (Jooß, 2013). It should be noted that the limit of quantification (LOQ) was defined in at lowest fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However, it should be mentioned, the LOQ of the original method (Jooß, 2013) is 0.05 mg/kg zoxamide.*

Reference:	KCP 5.1.2 (f)/31
Report	Parsons, Ch., 2020: Zoxium 240 SC– A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, BioChem agrar, Germany, Report No. GOW-17-13, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial standard soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% CaCO3 and 20% water content from the biological lab of Bio-Chem agrar was used. This has no influence on the integrity of the study results. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to determine the zoxamide concentrations in soil specimens. The determination of zoxamide (sum) was conducted by a method validated according to SANCO/825/00 rev. 8.1 and SANCO/3029/99 by PTRL. The method was provided by the Sponsor (Jooß, 2013). It was adapted to the equipment available at BioChem agrar. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide. Actually, the LOQ as determined in the original method validation (Jooß, 2013) is 0.5 mg/kg zoxamide.

5 g soil (weighed to ±0.05 g) was weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 10 µL of the acetonitrile phase were transferred to autosampler vials and diluted with 990 µL 0.125% formic acid in water (v/v). The analyte was determined after extraction with two mass transitions (m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

LC-MS System: Shimadzu LC20 HPLC system, Shimadzu 8040 LC-MS/MS detector.
Column: ACE Excel 3µm C18-AR 100 * 2.1 mm
Mobile phase: A: water containing 1mL/L formic acid
B: acetonitrile containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0	50	50
4	20	80
7	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI positive
Detection: m/z 336 → m/z 159 (quantifier)
m/z 336 → m/z 187 (qualifier)

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

Results and discussions

Table A 86: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1000	n.d.		n.d.		n.d.	
zoxamide	168.6	1000	188.8	112	167.8	100	165.4	98

Table A 87: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1250	n.d.		n.d.		n.d.	
zoxamide	210.8	1250	236.0	112	209.8	100	206.8	98

Recovery findings

Table A 88: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	1000	19.03	18.50	97	3.4
High	5	1000	190.3	188.7	99	3.0
Concentration in dry soil						

Low	5	1250	23.79	23.13	97	3.4
High	5	1250	237.9	235.9	99	3.0

Accuracy and precision / repeatability

The accuracy of the method, reported as mean recovery ± relative standard deviation, is shown in the table above.

Repeatability data was generated from five samples of test media fortified at the LOQ and five samples fortified at the highest nominal concentration of the test samples. The relative standard deviations (RSD) obtained at each fortification level were within the guideline requirements.

Linearity

The calibration was slightly non-linear, a quadratic fit with 1/c weighting was used in the range of 15.1 to 229 µg/L. This covers the range from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with a correlation coefficient R greater than 0.99. Accuracy was maintained by the measurement of recalibration standards before and after sample measurements.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However, the LOQ of the original method (Jooß, 2013) is 0.05 mg/kg zoxamide.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 106 days. The freezer storage stability of zoxamide residues in soil samples was confirmed for 643 days (21 months) in study no. 18 48 FEW 0001.

Stability of sample extracts

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Table A 89: Characteristics of the analytical method validation for the determination of zoxamide in artificial soil

	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data and calibration curve equations are presented in the study report. Example equation: $y = -1.923\ x^2 + 6565.17\ x + 4540.73$, $r^2 = 0.9999995$

	Zoxamide
Calibration range	15 to 229 µg/L in analytical samples (15 to 229 mg/kg in soil)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/quantification	19 mg/kg a.s. in moist soil (as received), corresponding to 24 mg/kg a.s. in dry soil and 19 µg/L a.s. in the analytical sample.

The following figure shows typical chromatograms.

Figure A 28: Chromatogram of lowest (above) and highest (below) standard solution

Conclusion

A method for the determination of zoxamide in soil was successfully (re-)validated according to SANCO/3029/99 (2000).

The nominal initial concentration in the fresh soil specimen could be confirmed – the recovery was greater than 80% (112%). The zoxamide concentrations stayed within the range of 98 – 112% of nominal throughout the whole study period.

(Parsons Ch. 2020)

Method for Zoxamide in artificial soil

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The method was re-validated with untreated artificial soil, equivalent to the soil used in the biological part of study. The limit of quantification (LOQ) was defined at the lowest fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However it should be mentioned, the original method was validated at LOQ of 0.05 mg/kg (zoxamide in dry soil) and at 10xLOQ (Jooß, 2013).*

Reference:	KCP 5.1.2 (f)/32
Report	Parsons, Ch., 2020: Zoxium 240 SC – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) in an artificial soil substrate Gowan Crop Protection Ltd., UK Mambo-Tox Ltd., UK, BioChem agrar, Germany, Report No. GOW-17-14, GLP, Not Published
Guideline(s):	SANCO/3029/99 rev. 4 (2000)
Deviations:	According to the analytical phase plan, the control samples should be used for validation. An equivalent artificial standard soil with 5% peat, 20% clay, 74.7% quartz sand, 0.3% $CaCO_3$ and 20% water content from the biological lab of BioChem agrar was used. This has no influence on the integrity of the study results. Reason for deviation: The quantities of the samples were too small to prepare all necessary validation samples.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this phase of the study was to confirm the applied test item concentrations in soil specimens. The active ingredient zoxamide was analysed by a validated method (Jooß, 2013). Actually, the LOQ of the original method is 0.05 mg/kg soil d.w. However, the method was adapted to the expected concentration range of this study and to the equipment available. It was re-validated with artificial soil substrate (5% peat, 20% clay, 74.7% quartz sand, 0.3% $CaCO_3$, wetted to 20% water content) spiked with test item at concentrations of 19 mg/kg a.s. in moist soil (24 mg/kg a.s. or 113 mg/kg test item in dry soil) and at 190 mg/kg a.s. in moist soil (238 mg/kg a.s. or 1129 mg/kg test item in dry soil) with 5 replicates per fortification level.

This amounts to a working range of approximately 25 - 110% of the test concentration. Additionally, two specimens were kept untreated as blank controls. All changes to the original method were validated according to SANCO/3029/99 rev. 4 prior to the test item measurements.

5 g soil (weighed to ±0.05 g) was weighed into a 100 mL Erlenmeyer flask. 1.0 mL water and 50 mL acetonitrile were added and the flasks were shaken for 45 minutes on a mechanical shaker. Then 1.0 g sodium chloride was added and the flasks again shaken for 10 minutes. The samples were transferred to centrifuge tubes and centrifuged for 3 minutes. Aliquots of 10 µL of the acetonitrile phase were transferred to autosampler vials and diluted with 990 µL 0.125% formic acid in water (v/v). The analyte was determined after extraction with two mass transitions (m/z 336 → 187 and 336 → 159), one for quantification and one for qualification, respectively.

Equipment

LC-MS System: Shimadzu LC20 HPLC system, Shimadzu 8040 LC-MS/MS detector.
Column: ACE Excel 3µm C18-AR 100 * 2.1 mm
Mobile phase: A: water containing 1mL/L formic acid
B: acetonitrile containing 1 mL/L formic acid

Time [min]	Solvent A [%]	Solvent B [%]
0.00	50	50
4.00	20	80
7.00	20	80
7.01	50	50

Flow rate: 0.4 mL/min
Run time: 10 min
Ionisation: ESI positive
Detection: m/z 336 → m/z 159 (quantifier)
m/z 336 → m/z 187 (qualifier)

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

Results and discussions

Table A 90: Analysis results in moist soil

Treatment group	Nominal concentration [mg/kg]	Sample preparation factor (mL/g)	Day 0		Day 14		Day 28	
			Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	1000	n.d.		n.d.		n.d.	
zoxamide	168.6	1000	171.4	102	180.2	107	184.6	109

Table A 91: Analysis results in dry soil

Treatment group	Nominal concentration [mg/kg]	Day 0		Day 14		Day 28	
		Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]	Analysed concentration [mg/kg]	Recovery [%]
Control	0.0	n.d.		n.d.		n.d.	
zoxamide	210.8	214.3	102	225.3	107	230.8	109

Recovery findings

Table A 92: Re-validation results for zoxamide

Validation	Replicates	Sample preparation factor [mL/g]	Nominal concentration [mg/kg]	Mean analysed concentration [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Concentration in moist soil						
Low	5	1000	19.03	18.50	97	3.4
High	5	1000	190.3	188.7	99	3.0
Concentration in dry soil						
Low	5	1250	23.79	23.13	97	3.4
High	5	1250	237.9	235.9	99	3.0

Accuracy and precision / repeatability

The accuracy of the method, reported as mean recovery ± relative standard deviation, is shown in the table below.

Repeatability data was generated from five samples of test media fortified at the LOQ and five samples fortified at the highest nominal concentration of the test samples. The relative standard deviations (RSD) obtained at each fortification level were within the guideline requirements.

Linearity

The calibration was slightly non-linear, a quadratic fit with 1/c weighting was used in the range of 15.1 to 229 µg/L. This covers the range from at least 80 % of the LOQ to at least 20 % above the highest nominal concentration levels, with a correlation coefficient R greater than 0.99. Accuracy was maintained by the measurement of recalibration standards before and after sample measurements.

Limit of quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level, i.e. 19 mg/kg zoxamide, in moist soil, equivalent to 24 mg/kg in dry soil and 19 µg/L in diluted extracts. However, the LOQ of the original method (Jooß, 2013) is 0.05 mg/kg zoxamide.

Matrix effects

Matrix effects were assessed by evaluating the recovery results from the spiked samples. Because of the high dilution with solvent, effects were not observed. Therefore, calibration solutions in solvent and no matrix-matched standards were used for quantification of zoxamide in the diluted sample extracts.

Specificity

The method is regarded as highly specific. It uses liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at two ion mass transitions for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention time as the analytes, were either non-detectable or amounted to less than 30 % of the limit of quantification (LOQ).

Storage stability of frozen samples

The soil samples were stored frozen for a maximum of 107 days. The freezer storage stability of zoxamide residues in soil samples was confirmed for 643 days (21 months) in study no. 18 48 FEW 0001.

Stability of sample extracts

Sample extracts and solvent standards were stored for less than 24 hours in the refrigerator or the cooled autosampler. Therefore, storage stability experiments of sample extracts and solvent standards are not applicable.

Table A 93: Characteristics of the analytical method validation for the determination of zoxamide in artificial soil

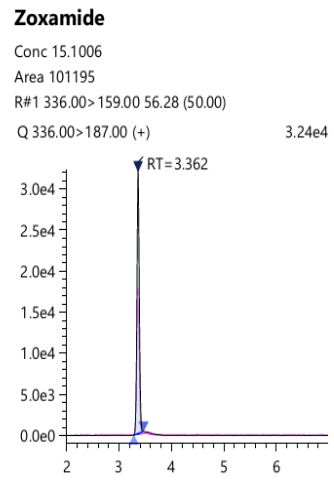
	Zoxamide
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30 % LOQ)
Calibration (type, number of data points)	5-point calibration with external standard Individual calibration data presented in section 7.1. Calibration curve equations: Example equation: $y = -1.923\ x^2 + 6565.17\ x + 4540.73$, $r^2 = 0.9999995$
Calibration range	15 to 229 µg/L in analytical samples (15 to 229 mg/kg in soil)
Assessment of matrix effects is presented	Recoveries of validation samples within 70-110%. No interfering peaks (validation blank samples had no peaks >30% of the lowest validation samples).
Limit of determination/quantification	19 mg/kg a.s. in moist soil (as received), corresponding to 24 mg/kg a.s. in dry soil and 19 µg/L a.s. in the analytical sample.

Following figure shows typical chromatograms.

17X24-25-Cal 1

Sample ID:
Date acquired: 15.11.2018 18:23:50
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_04.lcd
Vial: 2 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	2	Standard	15.11.2018 18:23:50	3.362	101195	32142	15.1006	99.95	56.28



17X24-25-Cal 5

Sample ID:
Date acquired: 15.11.2018 19:48:15
Acquired by: Hartmut Thomas
Data File: D:\2018\18CRX24-25 Zoxamid\17x24-25-1115_12.lcd
Vial: 6 | Inj. Volume: 2.0000uL | Tray: 1

Name	Vial	Sample Type	Acquired Date	Found RT	Area	Height	Conc.	Accuracy(%)	Ref 1 Actual Ratio
Zoxamide	6	Standard	15.11.2018 19:48:15	3.361	1383807	430928	229.7445	100.36	56.07

Zoxamide

Conc 229.7445
Area 1383807
R#1 336.00>159.00 56.07 (50.00)
Q 336.00>187.00 (+) 4.34e5

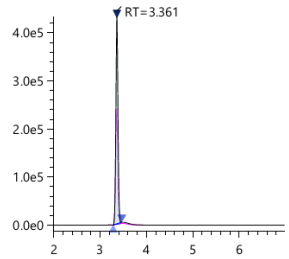


Figure A 29: Chromatogram of lowest (above) and highest (below) standard solution

Conclusion

A method for the determination of zoxamide in soil was successfully (re-)validated according to SANCO/3029/99 (2000).

The nominal initial concentration in the fresh soil specimen could be confirmed – the recovery was greater than 80% (102%). The zoxamide concentrations stayed within the range of 102 – 109% of nominal throughout the whole study period.

(Parsons Ch. 2020)

Method in support of a study on cleaning efficiency

The study investigated the cleaning efficiency of a formulation containing Zoxamide and Benalaxyl-M. The following summary focuses on the method validation results for Zoxamide.

Comments of zRMS:	The study has been accepted. The analytical method was based on HPLC-UV detection with matrix-matched standard solutions. The validity parameters were as required. 2 concentration levels (LOQ and high level) with 5 replicates at each level plus 2 blank samples were assessed. The mean recoveries ranged from 99% to 103% for the fortification levels and the precision RSD ≤20%. Thus, it could be concluded that the analytical method is suitable to demonstrate that residues after use of GWN-10392, a SC formulation containing 225 g/L zoxamide and 150 g/L benalaxyl-m do not remain in the spray tank after the recommended cleaning procedure.

	KCP 5.1.2 (g)/01
Reference:	
Report:	GWN-10392: EFFECTIVENESS OF CLEANING PROCEDURES, Fieseler, A., 2022, report No. 164781361, Doc. No. 247-001
Guideline(s):	OECD No. 302 (2001)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples taken from spray tank were diluted with acetonitrile / water (90/10) and analysed by HPLC-UV.

Chromatographic conditions – Zoxamide

System	HPLC-UV
Column	US ES RP 18 (250 * 4 mm) or equivalent
Mobile phase	80% methanol 20% water
Detection wavelength	220 nm
Retention time	Approx. 6.6 min

Results and discussions

The mean recovery at each fortification level were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 94: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/L) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water	Zoxamide	1.3 (n = 5)	99	1.3	-
		2.1 (n = 5)	99	1.2	-
		Overall (n = 10)	99	1.2	

Table A 95: Characteristics for the method used for validation of Zoxamide residues in water

	Zoxamide
Specificity	blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 9
Calibration range	Accepted calibration range in concentration units: 0.1 to 5 mg/L Corresponding calibration range in mass ratio units for the sample: 0.2 to 10 mg/L
Assessment of matrix effects is presented	Yes, no significant matrix effects were found
Limit of determination/quantification	1.3 mg/L for Zoxamide

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in water.

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 Analytical method 1

A 2.1.2.1.1.1 Method validation

Comments of zRMS:	The validation has been accepted. The analysis was performed by LC/MS-MS following the validated method. 2 transitions were monitored. The validation results summary is as follows:

HPLC-MS/MS determination of GWN-8030 - validation results summary

Parameter	Result	SANTE/2020/12830 rev.1 limit
Matrix effect	- 20.2 % / significant	< ±20%
Calibration (matrix-matched)	Range: 0.4990 – 49.90 µg/L in solution Range: 0.002 – 0.2 mg/kg on sample (from 20 % of LOQ to 100 % above 10xLOQ)	At least from 30% of LOQ to at least 20% above the highest level
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	Residuals randomly distributed
Recovery and precision (repeatability)	See nested table below	LOQ level (0.01 mg/kg) recoveries 70 – 110% RSD ≤20% 10xLOQ level (0.1 mg/kg) recoveries 70 – 110% RSD ≤15% (limits more restrictive than the guideline requirements)
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline	LOQ: lowest validated level with sufficient recovery and precision
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ) signal/noise ratio higher than 3	LOD < 30% of LOQ
Selectivity and specificity	Verified: no interferences found in untreated samples in amounts higher than 30% of the LOQ (< LOD)	Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory SRM transition. Calibration data, recovery and precision in compliance with the requirements	Confirmation by monitoring at least 1 SRM transition, providing linearity, recovery, precision, selectivity
Stability of the analyte in the sample extract	102.5 % after 3 days in the dark at 5 ± 3°C	70-120%
Stability of the analyte in the standard solution	Stock solution at about 1000 mg/L is stable for 123 days if stored in the dark at 5 ± 3°C (mean peak area difference of the stored solution and a freshly prepared solution: 0.77%)	Mean peak area difference of the stored solution and a freshly prepared solution: < 10%
Parameter	**Result**	**SANTE 2017/10632 rev. 3 limit**
Extraction efficiency	The extraction efficiency of Zoxamide from high water commodity matrices using the extraction procedure adopted in this study has already been successfully demonstrated in the mentioned study BPL-STUDY-18-000085	Extraction efficiency considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the metabolism study differ no more than 30%

Level	Concentration (mg/kg)	Transition	% Recovery	% RSD
LOQ (n = 5)	0.01	Primary (336/186.9)	106.0	3.5
		Confirmatory (336/159)	106.1	3.2
10xLOQ (n = 5)	0.1	Primary (336/186.9)	96.8	2.9
		Confirmatory (336/159)	96.3	2.6
Overall (n = 10)	/	Primary (336/186.9)	101.4	5.7
		Confirmatory (336/159)	101.2	5.8

n = number of replicates

Reference:	KCP 5.2 (a)/01
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN APPLES, Longhi, D., 2021, report No. GLP-STUDY-21-53, Doc. No. 432-003
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were extracted twice with methanol/acetonitrile/water 40/40/20 (v/v/v) containing 0.2% formic acid and centrifuged. The combined extracts were diluted with the extraction solvent and centrifuged. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Mobile phase	Mobile phase A: LC-MS grade water with 0.1 % formic acid Mobile phase B: LC-MS grade acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.5 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 96: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Apple	Zoxamide 336 > 186.9	0.01 (n = 5)	106.0	3.5	-
		0.1 (n = 5)	96.8	2.9	-
		Overall (n= 10)	101.4	5.7	-
Apple	Zoxamide 336 > 159.0	0.01 (n = 5)	106.1	3.2	-
		0.1 (n = 5)	96.3	2.6	-
		Overall (n= 10)	101.2	5.8	-

Table A 97: Characteristics for the analytical method used for validation of Zoxamide residues in apple

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.499 – 49.9 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

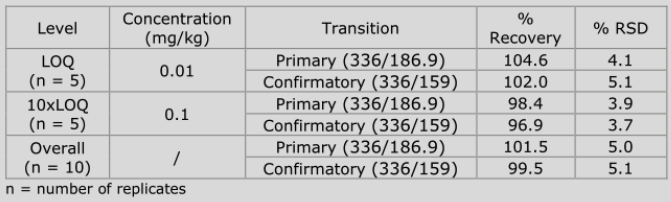
Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in plant matrices with high water content.

Comments of zRMS:	The validation has been accepted.

The objective of this study was the validation of the analytical method to determine Zoxamide in grape samples. The analytical determination was carried out using a LC-MS/MS method. The validation results summary is as follows:

HPLC-MS/MS determination of GWN-8030 - validation results summary

Parameter	Result	SANTE/2020/12830 rev.1
Matrix effect	- 17.25 % / not significant	< ±20%
Calibration (matrix-matched)	Range: 0.506 – 50.55 µg/L in solution Range: 0.002 – 0.2 mg/kg on sample (from 20 % of LOQ to 20xLOQ)	At least from 30% of LOQ to at least 20% above the highest level
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	Residuals randomly distributed
		LOQ level (0.01 mg/kg) recoveries 70 – 110% RSD ≤20% 10xLOQ level (0.1 mg/kg) recoveries 70 – 110% RSD ≤15% (limits more restrictive than the guideline requirements)
Limit of quantification (LOQ)	verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline	LOQ: lowest validated level with sufficient recovery and precision
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ) signal/noise ratio higher than 3	LOD < 30% of LOQ
Selectivity and specificity	Verified: no interferences found in untreated samples in amounts higher than 30% of the LOQ (< LOD)	Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory MS/MS transition. Calibration data, recovery and precision in compliance with the requirements	Confirmation by monitoring at least 1 additional MS/MS transition, providing linearity, recovery, precision, selectivity
Stability of the analyte in the sample extract	93.3 % (primary ion) and 92.8 % (confirmatory ion) after 3 days in the dark at 5 ± 3°C	70-120%
Stability of the analyte in the standard solution	Stock solution (1000 mg/L) is stable for 123 days if stored in the dark at 5 ± 3°C (mean peak area difference of the stored solution and a freshly prepared solution: 0.77%)	Mean peak area difference of the stored solution and a freshly prepared solution: < 10%
Parameter	**Result**	**SANTE 2017/10632 rev. 3**
Extraction efficiency	The extraction efficiency of Zoxamide in grape samples using the extraction procedure adopted in this study was successfully demonstrated in the study coded BPL-STUDY-18-000085	Extraction efficiency considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the metabolism study differ no more than 30%

Level	Concentration (mg/kg)	Transition	% Recovery	% RSD
LOQ (n = 5)	0.01	Primary (336/186.9)	104.6	4.1
		Confirmatory (336/159)	102.0	5.1
10xLOQ (n = 5)	0.1	Primary (336/186.9)	98.4	3.9
		Confirmatory (336/159)	96.9	3.7
Overall (n = 10)	/	Primary (336/186.9)	101.5	5.0
		Confirmatory (336/159)	99.5	5.1

n = number of replicates

Reference:	KCP 5.2 (a)/02
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF GWN-8030 IN GRAPES, Sala, A., 2022, report No. GLP-STUDY-21-101, Doc. No. 432-006
Guideline(s):	SANTE/2020/12830 rev. 1 (2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were extracted twice with methanol/acetonitrile/water 40/40/20 (v/v/v) containing 0.2% formic acid and centrifuged. The combined extracts were diluted with the extraction solvent and centrifuged. Analysis was carried out by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Mobile phase	Mobile phase A: LC-MS grade water with 0.1 % formic acid Mobile phase B: LC-MS grade acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.5 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 98: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Grape	Zoxamide 336 > 186.9	0.01 (n = 5)	104.6	4.1	-
		0.1 (n = 5)	98.4	3.9	-
		Overall (n= 10)	101.5	5.0	-
Grape	Zoxamide 336 > 159.0	0.01 (n = 5)	102.0	5.1	-
		0.1 (n = 5)	96.9	3.7	-
		Overall (n= 10)	99.5	5.1	-

Table A 99: Characteristics for the analytical method used for validation of Zoxamide residues in grape

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.506 – 50.55 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in plant matrices with high acid content.

A 2.1.2.1.2 Analytical method 2

Comments of zRMS:	The validation has been accepted. The analytical method was based on a solvent extraction and on an instrumental determination using a LC-MS/MS. The samples were extracted using a QuEChERS based method. The validation summary is as follows:

HPLC-MS/MS determination of Zoxamide in animal matrices – Validation results

Parameter	Result	SANTE/2020/12830 rev.1 limit
Matrix effect	Matrix: Oilseed rape seeds — Matrix effect (%): -37.2 (significant) Matrix: Wheat grain — Matrix effect (%): -16.4 (not significant)	< ±20%
Calibration (matrix-matched)	Matrix: Oilseed rape seeds, Wheat grain — Range (µg/L): 0.500 – 50.0 — Range: 0.0020 mg/kg – 0.200 mg/kg (20% LOQ – 100% above 10xLOQ)	At least from 30% of LOQ to at least 20% above the highest level
	The regressions residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	Residuals randomly distributed
Recovery and precision (repeatability)	**Oilseed rape seeds** Level: LOQ (n = 5) — Concentration: 0.01 mg/kg — Transition: Primary (336.0/186.9) — % Recovery: 88.8 — % RSD: 1.9 Transition: Confirmatory (336.0/159.0) — 87.4 — 2.8 Level: 10xLOQ (n = 5) — Concentration: 0.1 mg/kg — Primary (336.0/186.9) — 93.5 — 2.5 Confirmatory (336.0/159.0) — 91.6 — 2.2 Level: Overall (n = 10) — Concentration: / — Primary (336.0/186.9) — 91.1 — 3.4 Confirmatory (336.0/159.0) — 89.5 — 3.4 **Wheat grain** Level: LOQ (n = 5) — Concentration: 0.01 mg/kg — Primary (336.0/186.9) — % Recovery: 101.7 — % RSD: 2.4 Confirmatory (336.0/159.0) — 101.4 — 2.0 Level: 10xLOQ (n = 5) — Concentration: 0.1 mg/kg — Primary (336.0/186.9) — 102.0 — 1.6 Confirmatory (336.0/159.0) — 102.2 — 0.7 Level: Overall (n = 10) — Concentration: / — Primary (336.0/186.9) — 101.8 — 1.9 Confirmatory (336.0/159.0) — 101.8 — 1.4 n = number of replicates	LOQ level (0.01 mg/kg) recoveries 60 – 120% RSD ≤ 30% 10xLOQ level (0.1 mg/kg) recoveries 70 – 120% RSD ≤ 20%
Untreated sample	For each matrix: < 30% LOQ (< LOD, 2 replicates)	≤ 30% of LOQ
Limit of quantification (LOQ)	For each matrix: verified at 0.01 mg/kg recovery and repeatability data in compliance with the guideline	LOQ: lowest validated level with sufficient recovery and precision

HPLC-MS/MS determination of Zoxamide in animal matrices – Validation results

Parameter	Result	SANTE/2020/12830 rev.1 limit
Limit of detection (LOD)	Matrix: Oilseed rape seeds — Concentration: 0.0020 mg/kg Matrix: Wheat grain — Concentration: 0.0020 mg/kg signal/noise ratio higher than 3	LOD ≤ 30% of LOQ
Selectivity and specificity	Verified: no interferences found untreated samples in amounts higher than 30% of the LOQ (< LOD)	Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory SRM transition. Calibration data, recovery and precision in compliance with the requirements	Confirmation by monitoring at least 1 SRM transition, providing linearity, recovery, precision, selectivity
Stability of the analyte in the samples extract	Verified for at least 3 days at 5 ± 3°C in the dark, in detail: Matrix extract: Oilseed rape seeds — Storage conditions: 3 days at 5 ± 3°C — % Residual analyte after storage: -0.6 Matrix extract: Wheat grain — Storage conditions: 6 days at 5 ± 3°C — 3.7	70-120%
Stability of the analyte in the standard solution	Verified for 26 months at 4°C in the dark (stock solution in acetonitrile) in the GLP study coded BPL-STUDY-18-000091	< 10%

Reference: KCP 5.2 (a)/03

Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTIFICATION OF ZOXAMIDE IN HIGH OIL CONTENT AND DRY PLANT MATRICES, Longhi, D., 2022, report No. LBN-0001-2022, Doc. No. 432-002
Guideline(s):	SANTE/2020/12830 rev. 1 (2021), ENV/JM/MONO(2007)17
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples were extracted with acetonitrile after addition of water. A Quechers salt mixture (MgSO4, NaCl, trisodium citrate dehydrate, disodium hydrogen citrate sesquihydrate) was added, and the sample was centrifuged. The final extract was analysed by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Mobile phase	Mobile phase A: LC-MS grade water with 0.1 % formic acid Mobile phase B: LC-MS grade acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.2 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 100: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Oilseed rape	Zoxamide 336 > 186.9	0.01 (n = 5)	88.8	1.9	-
		0.1 (n = 5)	93.5	2.5	-
		Overall (n= 10)	91.1	3.4	-
Oilseed rape	Zoxamide 336 > 159.0	0.01 (n = 5)	87.4	2.8	-
		0.1 (n = 5)	91.6	2.2	-
		Overall (n= 10)	89.5	3.4	-
Wheat grain	Zoxamide 336 > 186.9	0.01 (n = 5)	101.7	2.4	-
		0.1 (n = 5)	102.0	1.6	-
		Overall (n= 10)	101.8	1.9	-
Wheat grain	Zoxamide 336 > 159.0	0.01 (n = 5)	101.4	2.0	-
		0.1 (n = 5)	102.2	0.7	-
		Overall (n= 10)	101.8	1.4	-

Table A 101: Characteristics for the analytical method used for validation of Zoxamide residues in oilseed rape and wheat grain

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.5 – 50.0 µg/L Corresponding calibration range in mass ratio units for the sample : 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12930 rev. 2 for the determination of Zoxamide in high fat matrices and dry matrices.

A 2.1.2.1.2.1 Independent laboratory validation

Comments of zRMS:	The ILV has been accepted. An independent validation of analytical methodology for the determination of residues of Zoxamide (GWN-8030) in different plant matrices (oilseed rape seeds, wheat grain, apples, grapes) has been performed. The validation parameters, linearity, matrix effects, specificity, selectivity, accuracy, precision, LOD, LOQ, stability of extracts and standard solutions were verified for Zoxamide in oilseed rape seeds, wheat grain, apples, and grapes samples. All the validation parameters were calculated for two mass transitions and were in compliance with the requirements.

Reference:	KCP 5.2 (a)/04
Report:	INDEPENDENT LABORATORY VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF GWN-8030 IN PLANT MATRICES, López Benet, F., 2023, report No. 435-22, Doc. No. 432-001
Guideline(s):	SANTE/2020/12830 rev. 1 (2021), OECD No. 39 (ENV/JM/MONO(2007)17), OPPTS 860.1340 (1996)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

There were no significant deviations from the primary method. Only. Two slight modifications were made: The flow rate was adjusted, and additional steps were added at the end of the chromatographic run to restore the initial conditions before the next injection.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex C18, 1.7 µm, 2.1 x 50 mm

Mobile phase	Mobile phase A: LC-MS grade water with 0.1 % formic acid Mobile phase B: LC-MS grade acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.2 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 102: Recovery results from independent laboratory validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Grape	Zoxamide 336 > 186.9	0.01 (n = 5)	82.2	3.5	
		0.1 (n = 5)	89.1	3.1	
		Overall (n= 10)	85.7	5.3	
Grape	Zoxamide 336 > 159.0	0.01 (n = 5)	82.9	6.2	
		0.1 (n = 5)	88.2	3.0	
		Overall (n= 10)	85.6	5.5	
Apple	Zoxamide 336 > 186.9	0.01 (n = 5)	86.9	3.8	-
		0.1 (n = 5)	100.7	2.7	-
		Overall (n= 10)	93.8	8.3	-
Apple	Zoxamide 336 > 159.0	0.01 (n = 5)	86.8	3.2	-
		0.1 (n = 5)	100.2	2.4	-
		Overall (n= 10)	93.5	8.0	-
Oilseed rape	Zoxamide 336 > 186.9	0.01 (n = 5)	74.4	2.1	-
		0.1 (n = 5)	89.8	3.3	-
		Overall (n= 10)	82.1	10.3	-
Oilseed rape	Zoxamide 336 > 159.0	0.01 (n = 5)	74.4	2.1	-
		0.1 (n = 5)	89.5	2.8	-
		Overall (n= 10)	81.9	10.0	-
Wheat grain	Zoxamide 336 > 186.9	0.01 (n = 5)	87.8	3.8	-
		0.1 (n = 5)	93.1	4.0	-
		Overall (n= 10)	90.4	4.8	-
Wheat grain	Zoxamide 336 > 159.0	0.01 (n = 5)	87.7	3.6	-
		0.1 (n = 5)	92.9	4.0	-
		Overall (n= 10)	90.3	4.7	-

Table A 103: Characteristics for the analytical method used for independent laboratory validation of Zoxamide residues in apple, grape, oilseed rape and wheat grain

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 7
Calibration range	Accepted calibration range in concentration units: 0.5 – 50 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002 – 0.2 mg/kg
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in plant matrices of high water, high acid, high fat content and dry matrices. The methods by Longhi, D. (2021), Report No. GLP-STUDY-21-101 and Longhi, D. (2022), Report No. GLP-STUDY-21-53 and LBN-0001-2022 were independently validated.

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The HPLC-MS/MS and HPLC-HRMS/MS method was acceptably validated for the analysis of zoxamide residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities. All mean accuracy and precision values are met requirements of SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. guidelines: mean recovery (accuracy) per level in the range 70-110 %, RSD% per level (precision) ≤ 20%. The limit of quantification (all analyte/matrix) is 0.01 mg/kg, accuracy and precision data at this level resulted in compliance with guidelines SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 complies SANTE/2020/12830, Rev.1. The LOQ for each enantiomer is 0.005 mg/kg. For metabolite RH-129151 (sum of R and S enantiomers) was used for validation due to lack of analyte.* *The extraction efficiency of the analytical method was acceptably verified according to SANTE 2017/10632 rev. 3.*

Reference:	KCP 5.2 (a)/05
Report:	VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE ZOXAMIDE RESIDUES IN GRAPE, POTATO, TOMATO, CUCUMBER, AND ONION RAW AGRICULTURAL AND PROCESSED COMMODITIES, Sala, A., 2020, report No. BPL-STUDY-18-000085, Doc. No. 432-009
Guideline(s):	SANCO/825/00 rev.8.1 (2010), SANCO/3029/99 rev. 4 (2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

Initially, a method has been developed on the basis of QuEChERS multi-residue method EN15662. However, this method was proven to be inadequate to fulfil the guideline's requirements and was therefore improved according to the specific needs, with an optimisation of the extraction solvent mixture and the extraction procedural steps. In particular to achieve a sufficient extraction efficiency the QuEChERS solvent (acetonitrile) was not adequate, several different solvent mixtures were tested to improve extraction efficiency and the final mixture taken forward was acetonitrile/ methanol/water 40/40/20 (v/v/v) containing 0.2% (v/v) formic acid, which achieved an optimal extraction efficiency when a 2-step extraction procedure was applied. After optimisation of the method, the extraction efficiency on all matrices/analytes resulted in comparable or better values than the corresponding extraction procedures applied in the plant metabolism studies. The final method can be hence considered an advanced and enhanced version of the starting reference EN15662 method.

The method developed allowes the determination of zoxamide and its mtetabolites via HPLC-MS/MS and HPLC-HRMS/MS in grape fruit, potato tuber, tomato fruit, cucumber fruit, and onion bulb raw agricultural and relevant processed commodities. It has been fully validated in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The extraction efficiency of the analytical method has been verified according to SANTE 2017/10632 rev. 3.

The homogenised specimen material (12.5 g each; 5 g in case of potato flakes) was extracted with a mixture of water/acetonitrile/methanol (20/40/40 v/v) acidified with 0.2% of HCOOH. The extraction was repeated twice on all samples except liquids (wine and grape juice); these matrices were analysed by direct injection after dilution with the extraction mixture. The 2 extracted fractions were pooled and combined and brought up to a final volume of 40 mL (20 mL for potato flakes) using the same solvent mixture. After centrifugation, the (purified) extracts were transferred to a glass vial for analyses using 2 HPLC/MS systems: a triple quadrupole HPLC-MS/MS and a high resolution HPLC-Orbitrap mass spectrometer. Typical HPLC conditions as well as typical R_f-values and MS spectra are summarised below.

It is possible to determine the analytes zoxamide (sum, R/S), RH-150721 (sum, R/S), RH-129151 (sum, R/S), RH-141288 (sum, R/S), RH-127450 (sum, R/S), RH-149736, RH-149737, RH-24549, RH-139432, RH-141452 and RH-141455.

All 5 pairs of chiral analytes were separated to receive their single enantiomers by chiral chromatography.

For metabolite RH-129151 - due to the lack of single isomer analytical standards - it was impossible to attribute the absolute configuration (R) and (S) to the measured peaks. Therefore, the 2 enantiomers were assigned in the following as "RH-129151 A" (the first enantiomer eluted in the chromatographic runs) and "RH-129151 B" (the second enantiomer eluted in the chromatographic runs).

The metabolites RH-141452, RH-141255 and RH-149737, which are known to form sugar conjugates, can be determined after an additional alkaline hydrolysis step according to the procedure of Steinborn et al. (2017). This step is based on QuEChERS method for the simultaneous determination of acidic pesticides, their esters and conjugates following alkaline hydrolysis. It allows to quantitatively hydrolyse potential conjugates of zoxamide metabolites. Following, the recoveries with and without this hydrolysis step can be compared.

Equipment Method A – Determination of analytes with a chiral center (except RH-129151)

Instrument:	HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470
Column:	Agilent Poroshell 120 EC-C18 4µm 4.6 x 100 mm + Phenomenex Lux 5µm Amylose-2 250 x 4.6 mm
Mobile phase:	A: 2 mM of ammonium formate in LC-MS grade water B: 90% acetonitrile UPLC grade / 10% methanol Elution mixture composition (isocratic run): 40% mobile phase A, 60% mobile phase B
Flow rate:	1 mL/min

Column temp.: 40°C
Injection volume: 20 µL
Retention time: (R)-zoxamide: approx.16.9 min
(S)-zoxamide: approx. 15.4 min
(R)-RH-141288: approx. 8.6 min
(S)-RH-141288: approx. 8.0 min
(R)-RH-150721: approx. 13.1 min
(S)-RH-150721: approx. 16.1 min
(R)-RH-127450: approx. 11.8 min
(S)-RH-127450: approx. 12.8 min
Stop time: 22 min
Ionisation: ESI (electrospray ionisation)

Ion mode:

Analyte	Detection	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
(R)-Zoxamide	Primary	338	188.9	125	25
	Confirmatory	336	186.9		
(S)-Zoxamide	Primary	338	188.9	125	25
	Confirmatory	336	186.9		
(R)-RH-141288	Primary	320	206	125	13
	Confirmatory	318	204		
(S)-RH-141288	Primary	320	206	125	13
	Confirmatory	318	204		
(R)-RH-150721	Primary	320	188.9	125	21
	Confirmatory	318	186.9		
(S)-RH-150721	Primary	320	188.9	125	21
	Confirmatory	318	186.9		
(R)-RH-127450	Primary	302	159	135	45
	Confirmatory	302	187		25
(S)-RH-127450	Primary	302	159	135	45
	Confirmatory	302	187		25

Equipment Method B – Determination of RH-129151

This molecule is analysed both with triple quadrupole HPLC-MS/MS instrument and HPLC-HRMS orbitrap system, the 2 methods are reported hereunder:

HPLC-MS/MS (Triple quadrupole spectrometer)

Instrument: HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470
Column: Phenomenex Kinetex C18, 5µm, 2.1 x 50 mm + Waters Trefoil Amy1, 2.5 µm, 3 x 150 mm
Mobile phase: A: 0.1 % v/v formic acid in LC-MS grade water
B: 0.1% v/v formic acid in ethanol
Elution mixture composition (isocratic run): 24% mobile phase A, 76% mobile phase B
Flow rate: 0.35 mL/min
Column temp.: 40°C
Injection volume: 10 µL
Retention time: RH-129151 (A): approx. 7.4 min
RH-129151 (B): approx. 9.0 min

Stop time: 15 min
Ionisation: ESI (electrospray ionisation)

Ion mode:

Analyte	Detection	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
RH-129151 (A)	Primary	300	186	150	33
	Confirmatory	302	188		
RH-129151 (B)	Primary	300	186	150	33
	Confirmatory	302	188		

HPLC-HRMS (Orbitrap spectrometer)

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Phenomenex Kinetex C18, 5µm, 2.1 x 50 mm + Waters Trefoil Amy1, 2.5 µm, 3 x 150 mm
Mobile phase: A: 0.1 % v/v formic acid in LC-MS grade water
B: 0.1% v/v formic acid in ethanol
Elution mixture composition (isocratic run): 24% mobile phase A , 76% mobile phase B
Flow rate: 0.35 mL/min
Column temp.: 40°C
Injection volume: 8 µL
Retention time: RH-129151 (A): approx. 6.8 min
RH-129151 (B): approx. 8.2 min
Stop time: 15 min
Ionisation: Parallel Reaction Monitoring (PRM), positive

Ion mode:

Analyte	Detection	Precursor ion	Product ion	Collision energy (V)
RH-129151 (A)	Primary	300.0553	185.9872	40
	Confirmatory	302.0523	187.9842	
RH-129151 (B)	Primary	300.0553	185.9872	40
	Confirmatory	302.0523	187.9842	

Equipment Method C – Determination of non-chiral analytes (except onion and potato tuber and processed commodities)

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Waters Xselect HSS PFP, 3.5 µm, 4.6 x 150 mm
Mobile phase: Solvent A: 0.2% v/v formic acid in LC-MS grade water
Solvent B: 0.1% v/v formic acid in methanol

Time (minutes)	Solvent A%	Solvent B%
0	80	20
2	80	20
10	0	100
13	0	100
13.1	80	20
16	80	20

Flow rate: 0.6 mL/min
Column temp.: 30°C
Injection volume: 4 µL

Retention time: RH-141452: approx. 10.28 min
RH-24549: approx. 12.05 min
RH-139432: approx. 11.37 min
RH-149736: approx. 9.09 min
RH-149737: approx. 6.64 min
Stop time: 16 min
Ionisation: ESI (electrospray ionisation)

Ion mode:

Analyte	Detection	Parent and Fragment ions (m/z)	Fragment ions (m/Z)	Collision energy (V)	Polarity
RH-141452	Primary	218.9621 (SIM)		/	
	Confirmatory	218.9621	144.9617	20	
RH-24549	Primary	202.9672 (SIM)		/	
	Confirmatory	202.9672	158.9774	20	
RH-139432	Primary	203.9977 (SIM)		/	Positive
	Confirmatory	203.9977	186.9709	45	
RH-149736	Primary	219.9927 (SIM)		/	
	Confirmatory	219.9927	158.9761	45	
RH-149737	Primary	187.9675		/	Negative
	Confirmatory	189.9446		/	

The analyses of the hydrolysed samples for the determination of the total (free + conjugated) RH-141452 and RH-149737 were carried out with this analytical method (except potato - tuber and processed commodities - samples).

Equipment Method D - Determination of non-chiral analytes in onion and potato tuber and processed commodities

Instrument: HPLC Vanquish coupled with HRMS Orbitrap Q-Exactive Thermo Scientific
Column: Waters Xselect HSS PFP, 3.5 µm, 4.6 x 150 mm
Mobile phase: Solvent A: 0.2% v/v formic acid in LC-MS grade water
Solvent B: 0.1% v/v formic acid in methanol

Time (minutes)	Solvent A%	Solvent B%
0	80	20
2	80	20
10	0	100
13	0	100
13.1	80	20
16	80	20

Flow rate: 0.6 mL/min
Column temp.: 30°C
Injection volume: 4 µL
Retention time: RH-141455: approx. 9.29 min
RH-141452: approx. 10.28 min
RH-24549: approx. 12.05 min
Stop time: 16 min
Ionisation: ESI (electrospray ionisation), negative

Ion mode:

Analyte	Detection	Parent and Fragment ions (m/z)	Fragment ions (m/Z)	Collision energy (V)	Polarity
RH-141455	Primary	232.9414 (SIM)		/	Negative

	Confirmatory	232.9414	188.9516	20	
RH-141452	Primary	218.9621 (SIM)		/	
	Confirmatory	218.9621	144.9617	20	
RH-24549	Primary	202.9672 (SIM)		/	
	Confirmatory	202.9672	158.9774	20	

The analyses of the hydrolysed samples for the determination of the total (free + conjugated) RH-141452 and RH-141455 in onion and potato tuber and processed commodities were carried out with this analytical method.

Results and discussion

Table A 104: Recovery results - grape fruits

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	100.1	2.4	**100.6**	**1.79**
	0.1 (10x LOQ)	101.1	1.0		
(S)-Zoxamide	0.01 (LOQ)	99.2	0.9	**99.1**	**0.87**
	0.1 (10x LOQ)	98.9	1.0		
Zoxamide sum	0.01 (LOQ)	99.6	1.1	**99.8**	**0.78**
	0.1 (10x LOQ)	100.0	0.2		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	93.4	4.6	**95.8**	**4.22**
	0.1 (10x LOQ)	98.2	2.1		
(S)-Zoxamide	0.01 (LOQ)	94.3	1.3	**96.4**	**2.89**
	0.1 (10x LOQ)	98.4	2.4		
Zoxamide sum	0.01 (LOQ)	93.9	2.8	**96.1**	**3.39**
	0.1 (10x LOQ)	98.3	2.2		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	98.1	1.9	**99.3**	**2.26**
	0.1 (10x LOQ)	100.6	2.0		
(S)-RH-150721	0.01 (LOQ)	104.3	1.1	**104.8**	**1.24**
	0.1 (10x LOQ)	105.3	1.3		
RH-150721 sum	0.01 (LOQ)	101.1	1.1	**102.0**	**1.53**
	0.1 (10x LOQ)	102.9	1.5		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	100.0	2.8	**102.3**	**3.12**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	104.6	1.4		
(S)-RH-150721	0.01 (LOQ)	101.7	1.5	**103.4**	**2.17**
	0.1 (10x LOQ)	105.1	1.3		
RH-150721 sum	0.01 (LOQ)	100.8	1.7	**102.8**	**2.45**
	0.1 (10x LOQ)	104.8	1.1		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	91.6	2.1	**88.3**	**4.6**
	0.1 (10x LOQ)	85.0	3.1		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	87.3	4.4	**86.4**	**4.4**
	0.1 (10x LOQ)	85.5	4.6		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	91.4	2.2	**88.7**	**4.4**
	0.1 (10x LOQ)	86.1	4.3		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	105.5	3.3	**93.9**	**13.4**
	0.1 (10x LOQ)	82.4	3.7		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	93.0	1.6	**89.2**	**5.1**
	0.1 (10x LOQ)	85.4	3.3		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	95.0	2.0	**88.6**	**8.1**
	0.1 (10x LOQ)	82.3	4.2		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	79.1	6.4	**79.0**	**5.44**
	0.1 (10x LOQ)	78.9	5.0		
RH-129151 (B)	0.01 (LOQ)	79.9	6.2	**77.8**	**5.52**
	0.1 (10x LOQ)	75.7	3.2		
RH-129151 (sum)	0.01 (LOQ)	77.8	6.4	**78.7**	**5.68**
	0.1 (10x LOQ)	79.6	5.3		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	76.5	6.5	**78.4**	**6.53**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	80.3	6.2		
RH-129151 (B)	0.01 (LOQ)	76.5	9.6	**76.6**	**8.11**
	0.1 (10x LOQ)	76.7	7.5		
RH-159151 (sum)	0.01 (LOQ)	78.2	7.7	**77.2**	**6.38**
	0.1 (10x LOQ)	76.2	5.2		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	89.0	2.4	**90.6**	**2.65**
	0.1 (10x LOQ)	92.3	1.5		
(S)-RH-141288	0.01 (LOQ)	98.7	2.6	**99.6**	**2.05**
	0.1 (10x LOQ)	100.4	1.1		
RH-141288 (sum)	0.01 (LOQ)	93.7	2.4	**95.0**	**2.19**
	0.1 (10x LOQ)	96.2	1.0		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	86.2	2.0	**89.6**	**4.24**
	0.1 (10x LOQ)	93.0	1.2		
(S)-RH-141288	0.01 (LOQ)	98.3	3.7	**99.9**	**3.07**
	0.1 (10x LOQ)	101.4	1.3		
RH-141288 (sum)	0.01 (LOQ)	92.1	1.8	**94.6**	**3.08**
	0.1 (10x LOQ)	97.1	1.0		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	97.5	3.3	**100.2**	**3.73**
	0.1 (10x LOQ)	102.9	1.7		
(S)-RH-127450	0.01 (LOQ)	99.7	1.7	**102.5**	**3.36**
	0.1 (10x LOQ)	105.3	2.0		
RH-127450 (sum)	0.01 (LOQ)	98.6	2.2	**101.3**	**3.43**
	0.1 (10x LOQ)	104.1	1.8		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	100.3	0.7	**101.2**	**1.46**
	0.1 (10x LOQ)	102.0	1.5		
(S)-RH-127450	0.01 (LOQ)	100.4	1.7	**101.5**	**1.89**
	0.1 (10x LOQ)	102.7	1.4		
RH-127450 (sum)	0.01 (LOQ)	100.3	0.8	**101.3**	**1.51**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	102.4	1.4		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	104.5	3.3	**94.4**	**11.7**
	0.1 (10x LOQ)	84.2	3.2		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	100.3	4.0	**92.2**	**10.1**
	0.1 (10x LOQ)	84.1	4.4		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	78.0	4.6	**80.2**	**4.9**
	0.1 (10x LOQ)	82.4	3.8		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	80.6	3.2	**81.0**	**4.3**
	0.1 (10x LOQ)	81.3	5.5		

Table A 105: Recovery results in juice

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	106.4	1.3	**105.8**	**2.24**
	0.1 (10x LOQ)	105.1	3.0		
(S)-Zoxamide	0.01 (LOQ)	106.9	2.3	**106.7**	**1.87**
	0.1 (10x LOQ)	106.4	1.6		
Zoxamide sum	0.01 (LOQ)	106.6	0.8	**106.2**	**1.20**
	0.1 (10x LOQ)	105.8	1.5		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	107.3	2.1	**106.4**	**2.03**
	0.1 (10x LOQ)	105.5	1.7		
(S)-Zoxamide	0.01 (LOQ)	104.9	2.0	**106.4**	**2.11**
	0.1 (10x LOQ)	107.8	1.4		
Zoxamide sum	0.01 (LOQ)	106.1	0.9	**106.4**	**0.95**
	0.1 (10x LOQ)	106.6	1.0		
RH-150721: Quantifier transition 320 → 188.9					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
(R)-RH-150721	0.01 (LOQ)	100.8	2.9	**103.2**	**3.31**
	0.1 (10x LOQ)	105.5	1.8		
(S)-RH-150721	0.01 (LOQ)	101.9	2.0	**103.9**	**2.72**
	0.1 (10x LOQ)	105.9	1.7		
RH-150721 sum	0.01 (LOQ)	101.3	2.1	**103.5**	**2.81**
	0.1 (10x LOQ)	105.7	1.5		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	100.1	4.6	**103.7**	**4.80**
	0.1 (10x LOQ)	107.3	1.5		
(S)-RH-150721	0.01 (LOQ)	107.4	1.4	**104.5**	**3.65**
	0.1 (10x LOQ)	101.6	3.1		
RH-150721 sum	0.01 (LOQ)	103.7	2.7	**104.1**	**2.26**
	0.1 (10x LOQ)	104.5	1.9		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	98.8	0.9	**97.2**	**2.7**
	0.1 (10x LOQ)	95.7	3.0		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	94.8	4.0	**95.2**	**2.8**
	0.1 (10x LOQ)	95.6	1.2		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	105.0	1.3	**102.4**	**2.9**
	0.1 (10x LOQ)	99.7	0.7		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	101.3	1.5	**98.8**	**4.1**
	0.1 (10x LOQ)	96.4	4.7		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	105.3	2.4	**102.1**	**3.8**
	0.1 (10x LOQ)	98.9	1.4		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	104.9	2.5	**102.2**	**3.4**
	0.1 (10x LOQ)	99.4	1.4		
RH-129151: Quantifier transition 300.0553 → 185.9872					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-129151 (A)	0.01 (LOQ)	93.4	11.4	**98.4**	**10.35**
	0.1 (10x LOQ)	103.4	7.2		
RH-129151 (B)	0.01 (LOQ)	88.8	13.0	**96.9**	**13.02**
	0.1 (10x LOQ)	105.1	7.3		
RH-129151 (sum)	0.01 (LOQ)	93.9	8.8	**96.9**	**8.20**
	0.1 (10x LOQ)	99.9	7.2		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	94.4	6.4	**95.4**	**6.71**
	0.1 (10x LOQ)	96.3	7.6		
RH-129151 (B)	0.01 (LOQ)	92.0	8.1	**95.4**	**8.08**
	0.1 (10x LOQ)	98.7	7.1		
RH-159151 (sum)	0.01 (LOQ)	90.4	10.4	**96.1**	**10.35**
	0.1 (10x LOQ)	101.9	7.0		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	102.2	2.5	**101.7**	**1.85**
	0.1 (10x LOQ)	101.2	1.0		
(S)-RH-141288	0.01 (LOQ)	100.0	1.5	**101.5**	**2.50**
	0.1 (10x LOQ)	102.9	2.6		
RH-141288 (sum)	0.01 (LOQ)	101.1	1.9	**101.6**	**1.52**
	0.1 (10x LOQ)	102.0	1.1		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	101.9	1.4	**101.6**	**1.36**
	0.1 (10x LOQ)	101.4	1.4		
(S)-RH-141288	0.01 (LOQ)	101.2	3.3	**103.5**	**3.14**
	0.1 (10x LOQ)	105.7	0.4		
RH-141288 (sum)	0.01 (LOQ)	101.6	2.0	**102.5**	**1.71**
	0.1 (10x LOQ)	103.5	0.7		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	109.0	0.7	**108.8**	**0.97**
	0.1 (10x LOQ)	108.7	1.3		
(S)-RH-127450	0.01 (LOQ)	109.3	1.4	**108.7**	**1.49**
	0.1 (10x LOQ)	108.2	1.6		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-127450 (sum)	0.01 (LOQ)	109.1	0.9	**108.8**	**1.03**
	0.1 (10x LOQ)	108.4	1.2		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	105.7	1.6	**106.6**	**1.53**
	0.1 (10x LOQ)	107.6	0.8		
(S)-RH-127450	0.01 (LOQ)	104.5	1.1	**105.6**	**2.08**
	0.1 (10x LOQ)	106.8	2.3		
RH-127450 (sum)	0.01 (LOQ)	105.1	0.7	**106.1**	**1.38**
	0.1 (10x LOQ)	107.2	1.2		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	96.8	1.9	**96.6**	**1.7**
	0.1 (10x LOQ)	96.4	1.7		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	99.6	3.4	**98.0**	**3.1**
	0.1 (10x LOQ)	96.4	1.7		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	104.6	3.2	**102.2**	**3.4**
	0.1 (10x LOQ)	99.8	0.9		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	103.0	2.6	**101.6**	**2.7**
	0.1 (10x LOQ)	100.1	2.0		

Table A 106: Recovery results - wine

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	106.2	3.3	**105.9**	**2.58**
	0.1 (10x LOQ)	105.7	1.9		
(S)-Zoxamide	0.01 (LOQ)	101.4	5.1	**103.0**	**3.87**
	0.1 (10x LOQ)	104.7	1.6		
Zoxamide sum	0.01 (LOQ)	103.8	4.1	**104.5**	**3.05**
	0.1 (10x LOQ)	105.2	1.7		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	94.9	2.3	**100.0**	**5.79**
	0.1 (10x LOQ)	105.1	2.2		
(S)-Zoxamide	0.01 (LOQ)	98.5	1.3	**101.2**	**3.08**
	0.1 (10x LOQ)	104.0	1.2		
Zoxamide sum	0.01 (LOQ)	96.7	1.6	**100.6**	**4.40**
	0.1 (10x LOQ)	104.5	1.7		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	86.3	4.9	**96.6**	**9.06**
	0.1 (10x LOQ)	101.0	2.9		
(S)-RH-150721	0.01 (LOQ)	104.1	2.9	**105.7**	**2.88**
	0.1 (10x LOQ)	107.2	2.2		
RH-150721 sum	0.01 (LOQ)	95.1	3.3	**99.6**	**5.44**
	0.1 (10x LOQ)	104.1	2.4		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	90.0	3.0	**96.1**	**7.19**
	0.1 (10x LOQ)	102.2	2.6		
(S)-RH-150721	0.01 (LOQ)	99.8	2.2	**103.6**	**4.48**
	0.1 (10x LOQ)	107.5	2.4		
RH-150721 sum	0.01 (LOQ)	94.8	1.8	**99.8**	**5.59**
	0.1 (10x LOQ)	104.8	2.1		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	99.5	2.2	**98.8**	**3.2**
	0.1 (10x LOQ)	98.1	4.1		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	100.2	3.1	**100.1**	**3.0**
	0.1 (10x LOQ)	100.0	3.3		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	101.4	2.3	**100.4**	**2.8**
	0.1 (10x LOQ)	99.5	3.2		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	108.4	1.1	**102.2**	**6.7**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	96.0	2.8		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	103.4	2.0	**99.7**	**4.5**
	0.1 (10x LOQ)	96.0	2.9		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	102.1	5.5	**100.3**	**4.7**
	0.1 (10x LOQ)	98.5	3.2		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	96.8	3.2	**88.0**	**11.26**
	0.1 (10x LOQ)	79.2	5.3		
RH-129151 (B)	0.01 (LOQ)	96.7	3.9	**87.8**	**11.45**
	0.1 (10x LOQ)	78.8	4.6		
RH-129151 (sum)	0.01 (LOQ)	97.6	2.7	**87.8**	**12.29**
	0.1 (10x LOQ)	78.0	5.2		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	98.4	2.4	**87.7**	**13.43**
	0.1 (10x LOQ)	76.9	5.4		
RH-129151 (B)	0.01 (LOQ)	98.2	2.0	**88.0**	**12.55**
	0.1 (10x LOQ)	77.9	4.5		
RH-159151 (sum)	0.01 (LOQ)	97.5	2.8	**87.9**	**11.95**
	0.1 (10x LOQ)	78.3	4.5		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	98.6	0.8	**99.3**	**1.13**
	0.1 (10x LOQ)	100.0	1.1		
(S)-RH-141288	0.01 (LOQ)	97.5	5.9	**100.7**	**5.04**
	0.1 (10x LOQ)	103.8	0.8		
RH-141288 (sum)	0.01 (LOQ)	98.1	2.7	**100.0**	**2.68**
	0.1 (10x LOQ)	101.8	0.7		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	97.8	2.5	**98.9**	**2.34**
	0.1 (10x LOQ)	100.0	1.7		
(S)-RH-141288	0.01 (LOQ)	95.1	3.5	**98.8**	**5.15**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	102.4	3.7		
RH-141288 (sum)	0.01 (LOQ)	96.5	2.6	**98.8**	**3.34**
	0.1 (10x LOQ)	101.2	2.0		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	101.1	3.2	**104.0**	**3.91**
	0.1 (10x LOQ)	107.0	2.1		
(S)-RH-127450	0.01 (LOQ)	102.9	1.0	**104.8**	**2.29**
	0.1 (10x LOQ)	106.8	1.4		
RH-127450 (sum)	0.01 (LOQ)	102.0	1.5	**104.4**	**2.91**
	0.1 (10x LOQ)	106.9	1.7		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	103.1	2.0	**105.4**	**2.86**
	0.1 (10x LOQ)	107.7	1.7		
(S)-RH-127450	0.01 (LOQ)	104.7	3.1	**105.4**	**2.64**
	0.1 (10x LOQ)	106.1	2.2		
RH-127450 (sum)	0.01 (LOQ)	103.9	2.4	**105.4**	**2.43**
	0.1 (10x LOQ)	106.9	1.6		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	96.9	1.4	**96.7**	**3.5**
	0.1 (10xLOQ)	96.6	5.0		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	97.4	6.4	**97.6**	**5.1**
	0.1 (10x LOQ)	97.9	4.3		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	105.6	3.1	**103.5**	**3.7**
	0.1 (10x LOQ)	101.3	3.3		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	102.1	6.3	**102.3**	**4.3**
	0.1 (10x LOQ)	102.5	1.4		

Table A 107: Recovery results - raisins

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	91.4	4.8	**93.8**	**5.00**
	0.1 (10x LOQ)	96.2	4.2		
(S)-Zoxamide	0.01 (LOQ)	90.4	5.4	**94.4**	**6.39**
	0.1 (10x LOQ)	98.5	4.2		
Zoxamide sum	0.01 (LOQ)	90.9	4.3	**94.1**	**5.38**
	0.1 (10x LOQ)	97.3	4.1		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	89.6	5.4	**93.5**	**6.39**
	0.1 (10x LOQ)	97.4	4.4		
(S)-Zoxamide	0.01 (LOQ)	93.9	3.8	**95.8**	**4.31**
	0.1 (10x LOQ)	97.7	4.2		
Zoxamide sum	0.01 (LOQ)	91.7	4.2	**94.6**	**5.14**
	0.1 (10x LOQ)	97.5	4.3		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	92.1	4.7	**95.1**	**4.91**
	0.1 (10x LOQ)	98.1	3.0		
(S)-RH-150721	0.01 (LOQ)	97.9	1.7	**101.1**	**4.28**
	0.1 (10x LOQ)	104.3	3.5		
RH-150721 sum	0.01 (LOQ)	95.0	3.1	**98.1**	**4.45**
	0.1 (10x LOQ)	101.2	3.2		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	93.2	3.8	**95.5**	**4.13**
	0.1 (10x LOQ)	97.9	2.9		
(S)-RH-150721	0.01 (LOQ)	107.6	2.0	**104.9**	**3.41**
	0.1 (10x LOQ)	102.2	2.4		
RH-150721 sum	0.01 (LOQ)	100.3	2.7	**100.2**	**2.35**
	0.1 (10x LOQ)	100.0	2.2		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	85.6	4.1	**91.6**	**7.62**
	0.1 (10x LOQ)	97.6	2.8		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	84.8	3.1	**92.3**	**9.21**
	0.1 (10x LOQ)	99.8	3.9		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	83.1	6.9	**88.4**	**8.32**
	0.1 (10x LOQ)	93.8	4.5		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	92.0	7.2	**89.7**	**6.52**
	0.1 (10x LOQ)	87.3	5.0		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	76.9	7.9	**85.8**	**12.19**
	0.1 (10x LOQ)	94.6	3.9		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	90.4	5.5	**90.4**	**4.25**
	0.1 (10x LOQ)	90.4	3.1		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	76.9	9.3	**81.8**	**8.79**
	0.1 (10x LOQ)	84.0	8.4		
RH-129151 (B)	0.01 (LOQ)	76.5	10.6	**80.6**	**10.45**
	0.1 (10x LOQ)	84.7	8.5		
RH-129151 (sum)	0.01 (LOQ)	81.4	8.9	**83.2**	**8.47**
	0.1 (10x LOQ)	85.0	8.4		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	83.1	8.9	**84.6**	**8.55**
	0.1 (10x LOQ)	86.0	8.8		
RH-129151 (B)	0.01 (LOQ)	82.2	11.4	**83.6**	**8.83**
	0.1 (10x LOQ)	85.0	6.4		
RH-159151 (sum)	0.01 (LOQ)	79.4	10.8	**82.1**	**9.12**
	0.1 (10x LOQ)	84.8	6.8		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	97.7	2.4	**97.4**	**3.03**
	0.1 (10x LOQ)	97.1	3.8		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
(S)-RH-141288	0.01 (LOQ)	95.1	7.1	**95.7**	**6.10**
	0.1 (10x LOQ)	96.3	5.7		
RH-141288 (sum)	0.01 (LOQ)	95.5	3.9	**96.6**	**3.44**
	0.1 (10x LOQ)	96.7	3.3		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	82.0	4.1	**90.4**	**10.24**
	0.1 (10x LOQ)	98.8	2.2		
(S)-RH-141288	0.01 (LOQ)	93.1	3.2	**89.6**	**9.75**
	0.1 (10x LOQ)	96.1	8.0		
RH-141288 (sum)	0.01 (LOQ)	82.5	2.6	**90.0**	**9.39**
	0.1 (10x LOQ)	97.5	4.2		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	86.4	5.2	**92.6**	**8.52**
	0.1 (10x LOQ)	98.8	4.9		
(S)-RH-127450	0.01 (LOQ)	86.0	5.5	**93.2**	**9.46**
	0.1 (10x LOQ)	100.5	4.6		
RH-127450 (sum)	0.01 (LOQ)	86.2	5.3	**92.9**	**8.97**
	0.1 (10x LOQ)	99.7	4.7		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	87.0	5.9	**92.4**	**7.87**
	0.1 (10x LOQ)	97.7	4.7		
(S)-RH-127450	0.01 (LOQ)	85.8	6.3	**93.0**	**9.62**
	0.1 (10x LOQ)	100.2	4.7		
RH-127450 (sum)	0.01 (LOQ)	86.4	6.0	**92.7**	**8.68**
	0.1 (10x LOQ)	98.9	4.6		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	91.9	3.3	**94.4**	**4.47**
	0.1 (10x LOQ)	96.9	4.0		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	95.1	6.6	**97.3**	**5.58**
	0.1 (10x LOQ)	99.4	3.9		
RH-149737: Quantifier transition 187.9675 (SIM)					

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-149737	0.01 (LOQ)	74.7	6.6	**82.8**	**11.41**
	0.1 (10x LOQ)	91.0	3.7		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	76.5	9.4	**83.5**	**11.19**
	0.1 (10x LOQ)	90.4	5.4		

Table A 108: Recovery results - potato tubers

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	102.0	2.8	**104.9**	**3.5**
	0.1 (10x LOQ)	107.8	0.9		
(S)-Zoxamide	0.01 (LOQ)	108.2	0.9	**107.2**	**2.0**
	0.1 (10x LOQ)	106.3	2.5		
Zoxamide sum	0.01 (LOQ)	105.1	1.7	**106.1**	**1.83**
	0.1 (10x LOQ)	107.0	1.7		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	102.3	3.3	**105.8**	**4.1**
	0.1 (10x LOQ)	19.3	0.8		
(S)-Zoxamide	0.01 (LOQ)	107.1	2.3	**107.7**	**1.7**
	0.1 (10x LOQ)	108.3	0.5		
Zoxamide sum	0.01 (LOQ)	104.8	2.4	**106.8**	**2.55**
	0.1 (10x LOQ)	108.8	0.6		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	87.4	6.2	**85.3**	**5.6**
	0.1 (10x LOQ)	83.2	4.1		
(S)-RH-150721	0.01 (LOQ)	92.1	4.2	**92.1**	**4.7**
	0.1 (10x LOQ)	92.2	5.7		
RH-150721 sum	0.01 (LOQ)	89.8	4.8	**88.7**	**4.49**
	0.1 (10x LOQ)	87.6	4.3		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	87.1	5.3	**84.7**	**5.2**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	82.3	3.6		
(S)-RH-150721	0.01 (LOQ)	97.8	5.3	**94.7**	**6.1**
	0.1 (10x LOQ)	91.7	5.4		
RH-150721 sum	0.01 (LOQ)	92.5	5.1	**89.7**	**5.51**
	0.1 (10x LOQ)	86.9	4.2		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	91.2	3.1	**94.5**	**4.5**
	0.1 (10x LOQ)	97.8	2.2		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	88.1	0.6	**92.7**	**5.3**
	0.1 (10x LOQ)	97.2	1.7		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	84.3	1.7	**87.6**	**4.2**
	0.1 (10x LOQ)	90.8	1.7		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	89.5	2.7	**89.2**	**2.6**
	0.1 (10x LOQ)	88.9	2.9		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	72.7	3.1	**86.6**	**17.0**
	0.1 (10x LOQ)	100.5	1.3		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	74.7	5.7	**85.9**	**14.4**
	0.1 (10x LOQ)	97.2	3.0		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	100.9	3.1	**103.2**	**3.81**
	0.1 (10x LOQ)	105.5	3.2		
RH-129151 (B)	0.01 (LOQ)	102.1	1.5	**102.9**	**2.94**
	0.1 (10x LOQ)	103.6	4.0		
RH-129151 (sum)	0.01 (LOQ)	101.5	2.2	**103.0**	**2.20**
	0.1 (10x LOQ)	104.6	0.7		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	106.1	1.9	**105.5**	**1.66**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	104.8	1.4		
RH-129151 (B)	0.01 (LOQ)	106.2	1.1	**106.0**	**1.16**
	0.1 (10x LOQ)	105.9	1.3		
RH-159151 (sum)	0.01 (LOQ)	106.0	1.2	**105.7**	**1.13**
	0.1 (10x LOQ)	105.4	1.1		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	98.7	3.3	**100.4**	**3.5**
	0.1 (10x LOQ)	102.1	3.0		
(S)-RH-141288	0.01 (LOQ)	104.9	3.4	**105.1**	**2.3**
	0.1 (10x LOQ)	105.3	0.7		
RH-141288 (sum)	0.01 (LOQ)	101.6	2.8	**102.7**	**2.35**
	0.1 (10x LOQ)	103.7	1.5		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	103.0	2.1	**102.6**	**1.7**
	0.1 (10x LOQ)	102.3	1.2		
(S)-RH-141288	0.01 (LOQ)	106.1	3.6	**106.6**	**2.6**
	0.1 (10x LOQ)	107.2	1.2		
RH-141288 (sum)	0.01 (LOQ)	104.6	1.8	**104.6**	**1.46**
	0.1 (10x LOQ)	104.7	1.2		

Table A 109: Recovery results - potato flakes

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	91.2	3.0	**89.7**	**4.45**
	0.1 (10x LOQ)	88.2	5.5		
(S)-Zoxamide	0.01 (LOQ)	91.8	2.6	**90.7**	**4.53**
	0.1 (10x LOQ)	89.7	6.1		
Zoxamide sum	0.01 (LOQ)	91.5	1.6	**90.2**	**4.20**
	0.1 (10x LOQ)	89.0	5.8		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	93.2	3.9	**90.1**	**5.86**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	87.0	5.8		
(S)-Zoxamide	0.01 (LOQ)	90.8	3.5	**89.9**	**4.71**
	0.1 (10x LOQ)	88.9	6.0		
Zoxamide sum	0.01 (LOQ)	92.0	3.5	**90.0**	**5.04**
	0.1 (10x LOQ)	88.0	5.8		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	94.8	1.0	**89.3**	**7.39**
	0.1 (10x LOQ)	83.7	5.4		
(S)-RH-150721	0.01 (LOQ)	94.7	1.8	**89.9**	**6.76**
	0.1 (10x LOQ)	85.0	5.3		
RH-150721 sum	0.01 (LOQ)	94.8	1.1	**89.6**	**7.04**
	0.1 (10x LOQ)	84.4	5.3		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	95.3	1.0	**89.6**	**7.55**
	0.1 (10x LOQ)	84.0	5.6		
(S)-RH-150721	0.01 (LOQ)	103.8	1.2	**98.5**	**6.55**
	0.1 (10x LOQ)	93.3	5.1		
RH-150721 sum	0.01 (LOQ)	99.5	0.5	**94.0**	**6.99**
	0.1 (10x LOQ)	88.6	5.4		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	84.8	1.3	**81.9**	**5.20**
	0.1 (10x LOQ)	79.0	5.4		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.4	0.8	**84.9**	**11.11**
	0.1 (10x LOQ)	76.5	5.9		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	86.3	0.8	**82.2**	**6.34**
	0.1 (10x LOQ)	78.1	5.6		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	93.9	0.8	**85.2**	**11.25**
	0.1 (10xLOQ)	76.6	5.4		
RH-141455: Quantifier transition 232.9414 (SIM)					

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141455	0.01 (LOQ)	82.3	1.1	**77.2**	**8.41**
	0.1 (10x LOQ)	72.0	7.4		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	101.0	4.0	**86.2**	**18.77**
	0.1 (10x LOQ)	71.4	7.0		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	86.5	2.6	**86.4**	**2.13**
	0.1 (10x LOQ)	86.4	1.8		
RH-129151 (B)	0.01 (LOQ)	74.8	4.8	**81.0**	**8.79**
	0.1 (10x LOQ)	87.2	2.5		
RH-129151 (sum)	0.01 (LOQ)	80.8	3.3	**83.8**	**4.47**
	0.1 (10x LOQ)	86.8	1.5		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	77.9	3.6	**81.8**	**5.82**
	0.1 (10x LOQ)	85.6	0.8		
RH-129151 (B)	0.01 (LOQ)	73.3	3.9	**80.5**	**9.99**
	0.1 (10x LOQ)	87.7	3.1		
RH-129151 (sum)	0.01 (LOQ)	75.6	4.4	**81.1**	**7.76**
	0.1 (10x LOQ)	86.7	1.4		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	93.7	3.9	**90.1**	**6.53**
	0.1 (10x LOQ)	86.4	6.4		
(S)-RH-141288	0.01 (LOQ)	78.5	2.6	**75.3**	**5.73**
	0.1 (10x LOQ)	72.0	4.6		
RH-141288 (sum)	0.01 (LOQ)	86.3	2.6	**82.8**	**5.83**
	0.1 (10x LOQ)	79.4	5.3		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	85.2	4.2	**85.9**	**4.63**
	0.1 (10x LOQ)	86.6	5.4		
(S)-RH-141288	0.01 (LOQ)	74.7	2.7	**73.2**	**3.79**
	0.1 (10x LOQ)	71.7	3.9		
RH-141288 (sum)	0.01 (LOQ)	80.1	1.8	**79.7**	**3.12**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	79.4	4.3		

Table A 110: Recovery results - fried potatoes

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	80.3	2.5	**77.2**	**4.68**
	0.1 (10x LOQ)	74.1	1.8		
(S)-Zoxamide	0.01 (LOQ)	79.8	4.5	**77.3**	**4.76**
	0.1 (10x LOQ)	74.7	1.5		
Zoxamide sum	0.01 (LOQ)	80.0	3.5	**77.2**	**4.64**
	0.1 (10x LOQ)	74.4	1.6		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	78.4	2.6	**76.3**	**3.56**
	0.1 (10x LOQ)	74.2	1.6		
(S)-Zoxamide	0.01 (LOQ)	77.4	4.6	**76.6**	**3.48**
	0.1 (10x LOQ)	75.7	1.5		
Zoxamide sum	0.01 (LOQ)	77.9	3.5	**76.4**	**3.29**
	0.1 (10x LOQ)	74.9	1.4		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	84.0	2.5	**81.7**	**3.60**
	0.1 (10x LOQ)	79.3	1.4		
(S)-RH-150721	0.01 (LOQ)	83.1	2.7	**81.5**	**2.94**
	0.1 (10x LOQ)	80.0	1.6		
RH-150721 sum	0.01 (LOQ)	83.6	2.4	**81.6**	**3.21**
	0.1 (10x LOQ)	79.6	1.5		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	82.7	2.3	**81.5**	**2.42**
	0.1 (10x LOQ)	80.4	1.6		
(S)-RH-150721	0.01 (LOQ)	90.0	3.3	**88.2**	**3.40**
	0.1 (10x LOQ)	86.4	2.0		
RH-150721 sum	0.01 (LOQ)	86.3	2.8	**84.9**	**2.90**
	0.1 (10x LOQ)	83.4	1.8		

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	93.6	1.6	**86.5**	**8.8**
	0.1 (10x LOQ)	79.5	2.5		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.0	0.8	**86.1**	**8.6**
	0.1 (10x LOQ)	79.3	3.3		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	95.7	1.1	**88.7**	**8.5**
	0.1 (10x LOQ)	81.8	2.8		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	99.3	1.4	**89.1**	**12.2**
	0.1 (10x LOQ)	78.9	2.8		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	78.7	1.0	**78.5**	**1.6**
	0.1 (10x LOQ)	78.2	2.1		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	82.9	3.9	**77.9**	**7.4**
	0.1 (10x LOQ)	73.0	1.9		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	74.4	3.3	**75.5**	**5.73**
	0.1 (10x LOQ)	76.6	7.5		
RH-129151 (B)	0.01 (LOQ)	75.6	2.6	**75.1**	**6.10**
	0.1 (10x LOQ)	74.7	8.8		
RH-129151 (sum)	0.01 (LOQ)	75.0	2.9	**75.3**	**5.72**
	0.1 (10x LOQ)	75.6	8.0		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	71.4	1.8	**73.4**	**5.86**
	0.1 (10x LOQ)	75.4	7.2		
RH-129151 (B)	0.01 (LOQ)	70.6	0.5	**71.4**	**6.29**
	0.1 (10x LOQ)	72.3	9.1		
RH-129151 (sum)	0.01 (LOQ)	71.0	0.9	**72.4**	**5.94**
	0.1 (10x LOQ)	73.8	8.2		

Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	77.4	4.7	**77.5**	**3.79**
	0.1 (10x LOQ)	77.5	3.2		
(S)-RH-141288	0.01 (LOQ)	78.1	5.70	**78.0**	**3.93**
	0.1 (10x LOQ)	77.9	1.6		
RH-141288 (sum)	0.01 (LOQ)	77.8	4.2	**77.7**	**3.17**
	0.1 (10x LOQ)	77.7	2.2		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	72.5	4.5	**74.6**	**4.38**
	0.1 (10x LOQ)	76.7	1.9		
(S)-RH-141288	0.01 (LOQ)	82.9	3.3	**81.2**	**3.40**
	0.1 (10x LOQ)	79.6	2.0		
RH-141288 (sum)	0.01 (LOQ)	77.5	2.7	**77.8**	**2.29**
	0.1 (10x LOQ)	78.1	2.0		

Table A 111: Recovery results – onion bulbs

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	84.2	7.7	**83.1**	**5.46**
	0.1 (10x LOQ)	82.0	1.4		
(S)-Zoxamide	0.01 (LOQ)	85.2	7.6	**83.7**	**5.88**
	0.1 (10x LOQ)	82.1	3.2		
Zoxamide sum	0.01 (LOQ)	84.7	7.1	**83.4**	**5.32**
	0.1 (10x LOQ)	82.0	2.3		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	83.1	5.2	**82.6**	**3.79**
	0.1 (10x LOQ)	82.1	2.1		
(S)-Zoxamide	0.01 (LOQ)	82.8	5.6	**82.1**	**3.97**
	0.1 (10x LOQ)	81.5	1.5		
Zoxamide sum	0.01 (LOQ)	82.9	5.4	**82.4**	**3.85**
	0.1 (10x LOQ)	81.8	1.8		

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	81.7	6.3	**84.3**	**5.5**
	0.1 (10x LOQ)	87.0	2.2		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	92.6	4.9	**90.5**	**4.8**
	0.1 (10x LOQ)	88.3	3.6		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	74.1	10.8	**85.2**	**15.2**
	0.1 (10x LOQ)	96.3	2.6		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	76.1	5.1	**85.3**	**12.0**
	0.1 (10x LOQ)	94.5	3.0		

Table A 112: Recovery results - tomato fruits

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	103.0	5.8	**98.3**	**6.46**
	0.1 (10x LOQ)	93.7	1.5		
(S)-Zoxamide	0.01 (LOQ)	104.1	6.6	**98.2**	**7.91**
	0.1 (10x LOQ)	92.3	1.3		
Zoxamide sum	0.01 (LOQ)	103.5	5.8	**98.3**	**7.01**
	0.1 (10x LOQ)	93.0	1.4		
Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	102.6	6.4	**97.8**	**6.94**
	0.1 (10x LOQ)	92.9	1.7		
(S)-Zoxamide	0.01 (LOQ)	105.9	4.6	**98.2**	**8.94**
	0.1 (10x LOQ)	90.5	1.3		
Zoxamide sum	0.01 (LOQ)	104.3	5.3	**91.7**	**1.4**
	0.1 (10x LOQ)	97.7	1.3		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	97.0	3.9	**96.8**	**2.89**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	96.6	1.9		
(S)-RH-150721	0.01 (LOQ)	106.3	0.9	**106.2**	**1.74**
	0.1 (10x LOQ)	106.0	2.5		
RH-150721 sum	0.01 (LOQ)	101.6	2.0	**101.4**	**1.69**
	0.1 (10x LOQ)	101.3	1.5		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	99.4	3.7	**96.5**	**4.48**
	0.1 (10x LOQ)	93.6	3.0		
(S)-RH-150721	0.01 (LOQ)	93.6	3.9	**101.1**	**8.26**
	0.1 (10x LOQ)	108.6	1.8		
RH-150721 sum	0.01 (LOQ)	96.5	3.4	**98.8**	**3.62**
	0.1 (10x LOQ)	101.0	2.2		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	86.5	2.9	**85.7**	**2.3**
	0.1 (10x LOQ)	85.0	1.4		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	84.5	5.1	**86.3**	**4.6**
	0.1 (10x LOQ)	88.0	3.5		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	75.9	4.9	**82.0**	**8.6**
	0.1 (10x LOQ)	88.2	1.8		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	80.9	2.0	**82.8**	**2.9**
	0.1 (10x LOQ)	84.7	1.2		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	73.0	3.0	**79.9**	**9.3**
	0.1 (10x LOQ)	86.8	1.1		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	78.6	6.6	**80.9**	**5.5**
	0.1 (10x LOQ)	83.2	2.6		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	75.2	5.9	**73.3**	**6.31**

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	70.7	5.5		
RH-129151 (B)	0.01 (LOQ)	75.6	11.1	**73.6**	**8.75**
	0.1 (10x LOQ)	71.6	5.1		
RH-129151 (sum)	0.01 (LOQ)	75.4	6.2	**73.3**	**6.24**
	0.1 (10x LOQ)	71.2	5.2		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	74.6	8.2	**73.1**	**6.80**
	0.1 (10x LOQ)	71.6	5.0		
RH-129151 (B)	0.01 (LOQ)	76.4	8.0	**74.4**	**6.9**
	0.1 (10x LOQ)	75.5	4.9		
RH-129151 (sum)	0.01 (LOQ)	75.5	8.0	**73.8**	**6.82**
	0.1 (10x LOQ)	72.0	4.9		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	91.3	3.6	**92.2**	**2.81**
	0.1 (10x LOQ)	93.0	1.6		
(S)-RH-141288	0.01 (LOQ)	90.0	5.5	**91.5**	**4.33**
	0.1 (10x LOQ)	93.1	2.4		
RH-141288 (sum)	0.01 (LOQ)	90.7	4.5	**91.9**	**3.44**
	0.1 (10xLOQ)	93.0	1.6		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	87.5	3.4	**90.8**	**4.63**
	0.1 (10x LOQ)	94.2	1.5		
(S)-RH-141288	0.01 (LOQ)	89.5	4.8	**90.6**	**3.65**
	0.1 (10x LOQ)	91.7	2.1		
RH-141288 (sum)	0.01 (LOQ)	88.5	4.0	**90.7**	**3.90**
	0.1 (10x LOQ)	93.0	1.8		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	98.1	3.0	**96.5**	**3.13**
	0.1 (10x LOQ)	95.0	2.5		
(S)-RH-127450	0.01 (LOQ)	95.4	2.8	**95.0**	**2.30**
	0.1 (10x LOQ)	96.6	1.9		
RH-127450 (sum)	0.01 (LOQ)	96.8	2.5	**95.8**	**2.46**

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
	0.1 (10x LOQ)	94.8	2.2		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	99.0	3.0	**97.0**	**3.27**
	0.1 (10x LOQ)	95.0	2.0		
(S)-RH-127450	0.01 (LOQ)	94.4	3.8	**94.3**	**2.79**
	0.1 (10x LOQ)	94.2	1.7		
RH-127450 (sum)	0.01 (LOQ)	96.7	3.1	**95.7**	**2.67**
	0.1 (10x LOQ)	94.6	1.8		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	81.2	5.5	**84.7**	**5.8**
	0.1 (10x LOQ)	88.3	1.9		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	78.8	4.0	**85.7**	**8.8**
	0.1 (10x LOQ)	92.5	1.2		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	77.1	4.8	**80.6**	**5.5**
	0.1 (10x LOQ)	84.0	0.9		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	76.5	3.8	**78.1**	**3.5**
	0.1 (10x LOQ)	79.7	1.6		

Table A 113: Recovery results - canned tomatoes

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	95.1	1.8	**95.0**	**2.99**
	0.1 (10x LOQ)	94.8	4.1		
(S)-Zoxamide	0.01 (LOQ)	91.2	3.6	**92.3**	**3.93**
	0.1 (10x LOQ)	93.5	4.2		
Zoxamide sum	0.01 (LOQ)	93.2	2.2	**93.6**	**3.19**
	0.1 (10x LOQ)	94.1	4.1		
Zoxamide: Qualifier transition 336 → 186.9					

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
(R)-Zoxamide	0.01 (LOQ)	93.5	2.8	**94.1**	**3.77**
	0.1 (10x LOQ)	94.7	4.8		
(S)-Zoxamide	0.01 (LOQ)	92.3	2.2	**93.2**	**3.26**
	0.1 (10x LOQ)	94.1	4.0		
Zoxamide sum	0.01 (LOQ)	92.9	2.5	**93.7**	**3.48**
	0.1 (10x LOQ)	94.4	4.4		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	105.6	2.2	**105.3**	**2.41**
	0.1 (10x LOQ)	105.0	2.8		
(S)-RH-150721	0.01 (LOQ)	107.5	2.3	**108.3**	**3.19**
	0.1 (10x LOQ)	109.1	4.0		
RH-150721 sum	0.01 (LOQ)	106.7	1.4	**106.8**	**2.43**
	0.1 (10x LOQ)	107.0	3.4		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	99.3	2.7	**102.3**	**3.83**
	0.1 (10x LOQ)	105.2	2.2		
(S)-RH-150721	0.01 (LOQ)	110.5	0.8	**109.8**	**3.022**
	0.1 (10x LOQ)	109.2	4.4		
RH-150721 sum	0.01 (LOQ)	104.8	1.4	**106.0**	**2.53**
	0.1 (10x LOQ)	107.2	3.0		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	86.8	1.5	**86.8**	**4.00**
	0.1 (10x LOQ)	86.8	5.8		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	79.1	2.3	**84.1**	**7.33**
	0.1 (10x LOQ)	89.1	4.9		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	86.7	0.6	**89.1**	**3.89**
	0.1 (10x LOQ)	91.4	3.9		
RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	93.8	2.0	**91.2**	**4.28**
	0.1 (10xLOQ)	88.5	4.1		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	91.7	1.8	**91.6**	**3.21**
	0.1 (10x LOQ)	91.4	4.5		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	88.9	2.1	**88.4**	**3.32**
	0.1 (10x LOQ)	88.0	4.5		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	72.4	9.0	**71.4**	**7.7**
	0.1 (10x LOQ)	70.3	6.8		
RH-129151 (B)	0.01 (LOQ)	75.5	10.0	**72.9**	**8.9**
	0.1 (10x LOQ)	70.2	6.4		
RH-129151 (sum)	0.01 (LOQ)	73.9	9.4	**72.1**	**8.51**
	0.1 (10x LOQ)	70.2	6.6		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	76.2	8.8	**73.9**	**7.9**
	0.1 (10x LOQ)	71.6	6.0		
RH-129151 (B)	0.01 (LOQ)	75.0	8.0	**73.3**	**6.9**
	0.1 (10x LOQ)	71.6	5.4		
RH-129151 (sum)	0.01 (LOQ)	75.6	8.4	**73.6**	**7.39**
	0.1 (10x LOQ)	71.6	5.6		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	95.8	1.1	**93.3**	**4.21**
	0.1 (10x LOQ)	90.9	4.7		
(S)-RH-141288	0.01 (LOQ)	95.5	1.7	**91.6**	**4.73**
	0.1 (10x LOQ)	87.7	1.4		
RH-141288 (sum)	0.01 (LOQ)	95.6	1.2	**92.5**	**3.99**
	0.1 (10x LOQ)	89.3	2.3		
RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	95.1	3.8	**91.7**	**5.29**
	0.1 (10x LOQ)	88.3	3.8		
(S)-RH-141288	0.01 (LOQ)	99.9	4.0	**94.3**	**7.01**
	0.1 (10x LOQ)	88.6	1.6		

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141288 (sum)	0.01 (LOQ)	97.4	3.1	**93.0**	**5.62**
	0.1 (10x LOQ)	88.5	1.5		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	98.5	3.1	**99.0**	**3.51**
	0.1 (10x LOQ)	99.5	4.1		
(S)-RH-127450	0.01 (LOQ)	91.5	1.2	**93.0**	**3.86**
	0.1 (10x LOQ)	94.5	5.0		
RH-127450 (sum)	0.01 (LOQ)	95.0	1.7	**96.0**	**3.45**
	0.1 (10x LOQ)	97.0	4.6		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	95.9	3.3	**97.7**	**3.85**
	0.1 (10x LOQ)	99.6	3.8		
(S)-RH-127450	0.01 (LOQ)	93.5	1.8	**94.0**	**3.86**
	0.1 (10x LOQ)	94.4	5.4		
RH-127450 (sum)	0.01 (LOQ)	94.6	2.1	**95.8**	**3.64**
	0.1 (10x LOQ)	97.0	4.6		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	94.0	1.8	**93.5**	**3.17**
	0.1 (10x LOQ)	93.1	4.4		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	88.3	2.8	**90.0**	**3.61**
	0.1 (10x LOQ)	91.8	3.5		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	84.9	4.8	**88.5**	**5.73**
	0.1 (10x LOQ)	92.2	3.1		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	103.7	8.7	**97.3**	**9.73**
	0.1 (10x LOQ)	90.8	4.6		

Table A 114: Recovery results - cucumber

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
Zoxamide: Quantifier transition 338 → 188.9					
(R)-Zoxamide	0.01 (LOQ)	82.6	4.7	**83.0**	**3.40**
	0.1 (10x LOQ)	83.4	1.9		
(S)-Zoxamide	0.01 (LOQ)	84.4	1.1	**84.4**	**1.29**
	0.1 (10x LOQ)	84.3	1.6		
Zoxamide sum	0.01 (LOQ)	83.5	2.8	**83.7**	**2.17**
	0.1 (10x LOQ)	83.9	1.5		

Zoxamide: Qualifier transition 336 → 186.9					
(R)-Zoxamide	0.01 (LOQ)	87.6	3.0	**85.6**	**3.27**
	0.1 (10x LOQ)	83.6	1.3		
(S)-Zoxamide	0.01 (LOQ)	87.8	1.8	**86.2**	**2.67**
	0.1 (10x LOQ)	84.6	2.0		
Zoxamide sum	0.01 (LOQ)	87.7	2.2	**85.9**	**2.81**
	0.1 (10x LOQ)	84.1	1.4		
RH-150721: Quantifier transition 320 → 188.9					
(R)-RH-150721	0.01 (LOQ)	99.6	5.7	**100.7**	**5.54**
	0.1 (10x LOQ)	101.9	5.8		
(S)-RH-150721	0.01 (LOQ)	83.7	2.5	**92.2**	**9.97**
	0.1 (10x LOQ)	100.7	2.2		
RH-150721 sum	0.01 (LOQ)	91.6	3.0	**96.5**	**5.99**
	0.1 (10x LOQ)	101.3	3.0		
RH-150721: Qualifier transition 318 → 186.9					
(R)-RH-150721	0.01 (LOQ)	101.6	4.1	**102.3**	**4.60**
	0.1 (10x LOQ)	103.0	5.4		
(S)-RH-150721	0.01 (LOQ)	86.6	4.1	**93.6**	**8.33**
	0.1 (10x LOQ)	100.7	1.2		
RH-150721 sum	0.01 (LOQ)	94.2	3.3	**98.0**	**5.15**
	0.1 (10x LOQ)	101.8	3.2		
RH-139432: Quantifier transition 203.9977 (SIM)					
RH-139432	0.01 (LOQ)	97.0	3.6	**94.3**	**5.9**
	0.1 (10x LOQ)	91.6	6.7		
RH-139432: Qualifier transition 203.9977 → 158.9761					
RH-139432	0.01 (LOQ)	82.0	6.3	**88.4**	**9.1**
	0.1 (10x LOQ)	94.7	4.3		
RH-24549: Quantifier transition 202.9672 (SIM)					
RH-24549	0.01 (LOQ)	85.2	5.1	**89.0**	**6.8**
	0.1 (10x LOQ)	92.9	5.5		

RH-24549: Qualifier transition 202.9672 → 158.9774					
RH-24549	0.01 (LOQ)	91.7	3.6	**91.4**	**3.9**
	0.1 (10x LOQ)	91.0	4.6		
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	91.8	3.6	**92.2**	**4.4**
	0.1 (10x LOQ)	92.6	5.4		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	93.7	5.3	**90.9**	**6.3**
	0.1 (10x LOQ)	88.2	6.3		
RH-129151: Quantifier transition 300.0553 → 185.9872					
RH-129151 (A)	0.01 (LOQ)	90.2	11.7	**87.2**	**10.38**
	0.1 (10x LOQ)	84.2	8.3		
RH-129151 (B)	0.01 (LOQ)	96.9	9.4	**90.6**	**10.74**
	0.1 (10x LOQ)	84.2	6.4		
RH-129151 (sum)	0.01 (LOQ)	93.6	10.5	**88.9**	**10.34**
	0.1 (10x LOQ)	84.2	7.4		
RH-129151: Qualifier transition 300.0523 → 185.9842					
RH-129151 (A)	0.01 (LOQ)	94.2	10.0	**89.2**	**9.35**
	0.1 (10x LOQ)	86.0	7.9		
RH-129151 (B)	0.01 (LOQ)	100.5	6.5	**94.5**	**9.04**
	0.1 (10x LOQ)	88.5	6.3		
RH-129151 (sum)	0.01 (LOQ)	96.5	8.1	**91.9**	**8.94**
	0.1 (10x LOQ)	87.3	7.0		
RH-141288: Quantifier transition 320 → 206					
(R)-RH-141288	0.01 (LOQ)	80.7	4.6	**83.2**	**4.39**
	0.1 (10x LOQ)	85.6	1.2		
(S)-RH-141288	0.01 (LOQ)	81.8	2.8	**82.7**	**2.68**
	0.1 (10x LOQ)	83.6	2.2		
RH-141288 (sum)	0.01 (LOQ)	81.2	3.2	**82.9**	**3.22**
	0.1 (10x LOQ)	84.7	1.7		

RH-141288: Qualifier transition 318 → 204					
(R)-RH-141288	0.01 (LOQ)	76.3	1.3	**80.0**	**5.16**
	0.1 (10x LOQ)	83.7	2.0		
(S)-RH-141288	0.01 (LOQ)	75.6	3.1	**78.5**	**4.85**
	0.1 (10x LOQ)	81.4	3.0		
RH-141288 (sum)	0.01 (LOQ)	75.9	2.1	**79.3**	**4.91**
	0.1 (10x LOQ)	82.6	2.4		
RH-127450: Quantifier transition 302 → 159					
(R)-RH-127450	0.01 (LOQ)	74.7	3.4	**77.7**	**4.76**
	0.1 (10x LOQ)	80.7	1.8		
(S)-RH-127450	0.01 (LOQ)	81.3	1.4	**82.7**	**2.56**
	0.1 (10x LOQ)	84.0	2.4		
RH-127450 (sum)	0.01 (LOQ)	78.0	2.2	**80.2**	**3.47**
	0.1 (10x LOQ)	82.4	1.9		
RH-127450: Qualifier transition 302 → 187					
(R)-RH-127450	0.01 (LOQ)	75.7	2.0	**78.5**	**4.21**
	0.1 (10x LOQ)	81.4	1.7		
(S)-RH-127450	0.01 (LOQ)	85.2	3.4	**85.1**	**2.99**
	0.1 (10x LOQ)	85.0	2.9		
RH-127450 (sum)	0.01 (LOQ)	80.4	2.3	**81.8**	**2.77**
	0.1 (10x LOQ)	83.2	2.2		
RH-149736: Quantifier transition 219.9927 (SIM)					
RH-149736	0.01 (LOQ)	97.4	3.8	**98.4**	**3.9**
	0.1 (10xLOQ)	99.4	4.1		
RH-149736: Qualifier transition 219.9927 → 158.9761					
RH-149736	0.01 (LOQ)	102.2	3.2	**101.3**	**3.6**
	0.1 (10x LOQ)	100.3	4.1		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	89.3	11.1	**89.4**	**8.3**
	0.1 (10x LOQ)	89.5	5.5		

RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	88.4	11.8	**89.0**	**8.4**
	0.1 (10x LOQ)	89.6	4.7		

Table A 115: Recovery results - grape fruits – conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	106.5	1.0	**105.8**	**1.8**
	0.1 (10x LOQ)	105.0	2.2		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	87.2	1.0	**94.8**	**8.5**
	0.1 (10x LOQ)	102.4	1.1		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	109.4	1.7	**109.4**	**1.3**
	0.1 (10x LOQ)	109.4	1.1		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	98.0	4.0	**102.0**	**5.1**
	0.1 (10x LOQ)	106.1	2.2		

Table A 116: Recovery results - grape juice - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	106.4	2.2	**103.5**	**3.5**
	0.1 (10x LOQ)	100.6	1.7		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	104.0	2.9	**103.4**	**2.2**
	0.1 (10x LOQ)	102.7	1.3		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	108.0	3.3	**107.5**	**2.3**
	0.1 (1x LOQ)	107.0	0.9		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	107.5	2.0	**107.0**	**1.7**
	0.1 (10x LOQ)	106.5	1.3		

Table A 117: Recovery results - wine - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	94.6	4.3	**92.3**	**4.0**
	0.1 (10x LOQ)	90.1	1.0		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	81.8	3.8	**84.7**	**4.5**
	0.1 (10x LOQ)	87.6	1.7		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	106.8	2.1	**100.8**	**6.6**
	0.1 (10x LOQ)	94.8	2.3		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	107.1	2.1	**101.5**	**6.0**
	0.1 (10x LOQ)	96.0	1.1		

Table A 118: Recovery results - raisins - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	72.5	0.4	**72.1**	**0.8**
	0.1 (10x LOQ)	71.8	0.8		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	74.9	2.9	**72.6**	**3.95**
	0.1 (10x LOQ)	70.3	1.3		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	79.1	3.6	**92.8**	**15.76**
	0.1 (10x LOQ)	106.5	2.0		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	73.8	4.3	**86.6**	**15.97**
	0.1 (10x LOQ)	99.8	0.5		

Table A 119: Recovery results - potato tubers - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	93.3	6.5	**91.0**	**5.3**
	0.1 (10x LOQ)	88.6	1.5		
RH-141452: Qualifier transition 232.9414 → 188.9516					
RH-141452	0.01 (LOQ)	110.7	13.8	**100.7**	**14.9**
	0.1 (10x LOQ)	90.7	5.3		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	97.1	19.3	**92.6**	**14.6**
	0.1 (10x LOQ)	88.1	2.6		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	109.0	3.6	**99.3**	**10.7**
	0.1 (10x LOQ)	89.5	1.8		

Table A 120: Recovery results - potato flakes - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	99.2	3.3	**97.1**	**3.37**
	0.1 (10xLOQ)	95.0	1.7		
RH-141452: Qualifier transition 232.9414 → 188.9516					
RH-141452	0.01 (LOQ)	102.2	4.3	**99.8**	**4.31**
	0.1 (10x LOQ)	97.3	2.8		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	79.3	7.0	**77.4**	**5.46**
	0.1 (10x LOQ)	75.6	1.7		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	73.2	3.0	**71.7**	**3.23**
	0.1 (10x LOQ)	70.3	2.0		

Table A 121: Recovery results - fried potatoes - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	86.9	2.3	**85.1**	**2.8**
	0.1 (10x LOQ)	83.3	0.5		
RH-141452: Qualifier transition 232.9414 → 188.9516					
RH-141452	0.01 (LOQ)	95.1	1.4	**87.9**	**8.8**
	0.1 (10x LOQ)	80.7	1.8		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	71.9	3.9	**71.3**	**2.8**
	0.1 (10x LOQ)	70.7	0.1		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	76.6	4.1	**73.3**	**5.5**
	0.1 (10x LOQ)	70.1	1.0		

Table A 122: Recovery results - tomato fruits - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	72.6	1.6	**81.5**	**11.7**
	0.1 (10x LOQ)	90.4	2.7		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	81.8	1.9	**84.7**	**3.9**
	0.1 (10x LOQ)	87.6	1.6		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	75.8	2.8	**87.4**	**14.3**
	0.1 (10x LOQ)	99.1	2.5		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	74.3	3.9	**86.8**	**15.5**
	0.1 (10x LOQ)	99.4	2.0		

Table A 123: Recovery results - canned tomatoes - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	104.7	3.2	**104.0**	**2.41**
	0.1 (10x LOQ)	103.3	1.3		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	106.4	2.2	**102.6**	**4.22**
	0.1 (10x LOQ)	98.9	1.4		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	109.1	0.8	**108.4**	**0.92**
	0.1 (10x LOQ)	107.8	0.6		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	102.5	4.0	**105.7**	**4.21**
	0.1 (10x LOQ)	108.9	1.4		

Table A 124: Recovery results - cucumber - conjugates

Analyte	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall mean RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	82.6	14.0	**82.3**	**11.2**
	0.1 (10x LOQ)	82.0	9.3		
RH-141452: Qualifier transition 218.9621 → 144.9617					
RH-141452	0.01 (LOQ)	101.1	9.8	**90.2**	**15.9**
	0.1 (10x LOQ)	79.4	10.4		
RH-149737: Quantifier transition 187.9675 (SIM)					
RH-149737	0.01 (LOQ)	83.2	19.8	**85.2**	**14.0**
	0.1 (10x LOQ)	87.2	7.2		
RH-149737: Qualifier transition 189.9647 (SIM)					
RH-149737	0.01 (LOQ)	92.4	17.9	**90.7**	**13.1**
	0.1 (10x LOQ)	89.0	6.9		

Table A 125: Recovery results - onion bulbs - conjugates

Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall mean recovery (%)	Overall RSD (%)
RH-141452: Quantifier transition 218.9621 (SIM)					
RH-141452	0.01 (LOQ)	83.2	3.6	**82.2**	**3.3**
	0.1 (10x LOQ)	81.1	2.8		
RH-141452: Qualifier transition 232.9414 → 188.9516					
RH-141452	0.01 (LOQ)	97.4	5.5	**89.3**	**10.6**
	0.1 (10xL OQ)	81.2	2.9		
RH-141455: Quantifier transition 232.9414 (SIM)					
RH-141455	0.01 (LOQ)	87.0	3.8	**79.7**	**10.2**
	0.1 (10x LOQ)	72.3	1.5		
RH-141455: Qualifier transition 232.9414 → 188.9516					
RH-141455	0.01 (LOQ)	94.8	4.6	**82.9**	**15.5**
	0.1 (10x LOQ)	71.7	1.7		

Accuracy and repeatability/precision

Accuracy and precision were verified by means of recovery tests carried out at 2 spiking levels:
- 0.01 mg/kg (0.005 mg/kg for each single enantiomer) corresponding to the target LOQ
- 0.1 mg/kg (0.005 mg/kg for each single enantiomer) corresponding to 10 x LOQ

The mean recoveries per spiking level for both primary and confirmatory transition fulfil the guideline requirements (mean recovery per level in the range 70-110% and RSD per level < 20%).

Linearity

Linearity of response was verified as follows with matrix-matched standard solutions.
- 5-point calibration curves.
- Calibration ranges of 1 - 50 µg/L on final extract.
  Exceptions: for wine and grape juice samples, nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flake samples the lowest concentration was 0.7 µg/L (corresponding to 2.8 µg/kg in the sample).
- Range of 3 - 160 mg/kg per sample, corresponding to 30% LOQ – 50 x LOQ.
- For analytes with a chiral center: Considering the sum of 2 enantiomers.
- R^2 > values 0.99.
- Linearity always checked for 2 MRM (Multiple Reaction Monitoring) transitions in case of Triple Quadrupole mass analysis or for the 2 high resolution ions in case of HRMS-Orbitrap analysis.

Limit of quantification (all analyte/matrix)

LOQ = 0.01 mg/kg. For analytes with a chiral center this limit is referred to the sum of the 2 enantiomers

(i.e. the LOQ for each enantiomer is 0.005 mg/kg)

Limit of detection (all analyte/matrix)

LOD = 1 µg/L / 0.003 mg/kg on sample / 30% of LOQ (0.01 mg/kg).

The signal to noise ratio measured at LOD for all analyte/matrix was always higher than 3. Besides, for analytes with a chiral center this limit is referred to the sum of the 2 enantiomers (i.e. the LOD for each enantiomer is 0.0015 mg/kg).

Matrix effects

All calibration curves, for all matrices/analyte were prepared using matrix matched analytical standards. Blank sample extracts, prepared with the same procedure applied on spiked samples were used to dilute stock solutions. Using this approach, the matrix effect on all samples analysed was nullified.

Specificity

The methods are highly specific with characteristic R_f-values and the measurement of 2 MRM (Multiple Reaction Monitoring) transitions in case of Triple quadrupole mass analysis or 2 high resolution ions in case of HRMS-Orbitrap analysis per analyte.

For all analytes in all matrices the blank sample signal (for both for the primary and the confirmatory detection) was always < 30% LOQ, in most cases the signal in the blank samples was zero (i.e. no detectable/quantifiable at analyte retention time).

Stability of sample extracts

The stability of the analytes in the final extracts stored at around 4°C was confirmed by recovery experiments for a storage period of 3 days, corresponding to the maximum period of time elapsed between sample extraction and analysis. The stability of the extracts was considered proven if the difference between the instrument response for a freshly prepared spiked samples compared with the stored ones was ≤ ±20%.

The metabolite RH-129151 was the only analyte not stable in the final extract. Therefore, the analysis of this analyte in all samples needs to be and was always carried out immediately after extraction (analytical sequences in this study completed within 8 hours after sample extraction).

Table A 126: Characteristics for the analytical method used for validation of zoxamide and its metabolites in plant matrices

	Zoxamide (sum, R/S), RH-150721 (sum, R/S), RH-129151 (sum, R/S), RH-141288 (sum, R/S), RH-127450 (sum, R/S), RH-149736, RH-149737, RH-24549, RH-139432, RH-141452, RH-141455
Specificity	HPLC-MS/MS and HPLC-HRMS/MS are regarded as highly specific. Mass spectrum is provided. Blank value < 30 % LOQ
Calibration (type, number of data points)	Matrix-matched standard calibration. 5-point calibration; r^2 > 0.99; linear Calibration data and calibration line equation presented in the study report.
Calibration range	1-50 µg/L on final extract Exceptions: for wine and grape juice samples nominal concentration of standard range 3 - 120 µg/mL (corresponding to 3-120 µg/g in the sample), for flakes the lowest concentration was 0.7µg/L (corresponding to a concentration of 2.8 µg/kg and 3-160 mg/kg in the sample)
Assessment of matrix effects is presented	Yes
Limit of quantification (LOQ) Limit of detection (LOD)	LOQ: 0.01 mg/kg 0.005 mg/kg per enantiomer for substances with a chiral center LOD:

	0.003 mg/kg 0.0015 mg/kg per enantiomer for substances with a chiral center

The following figures show example chromatograms and mass spectra.

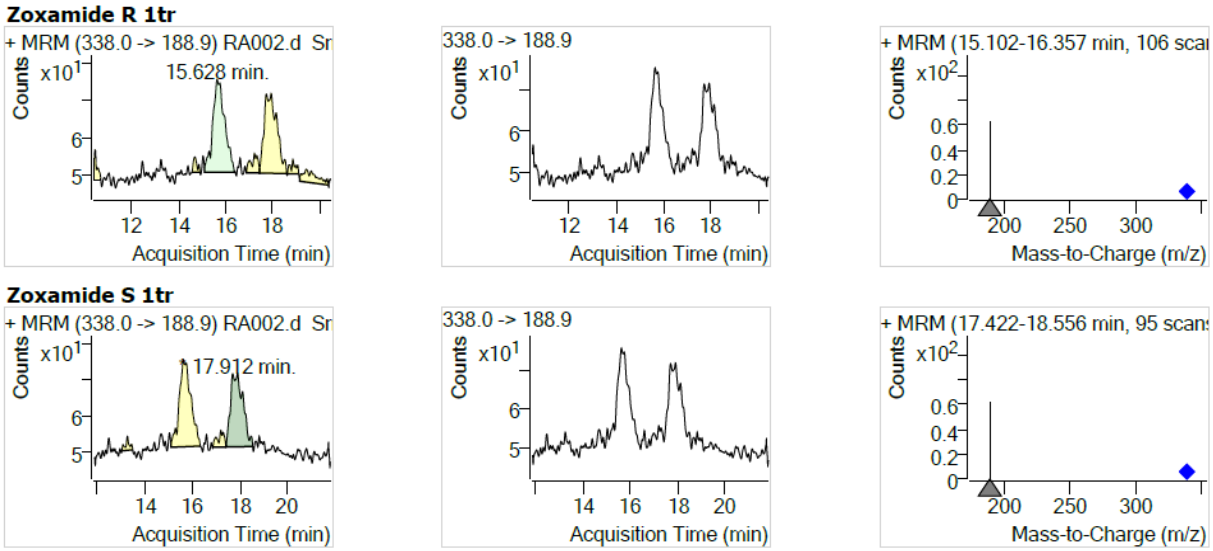


Figure A 30: Chromatogram of zoxamide (R/S)

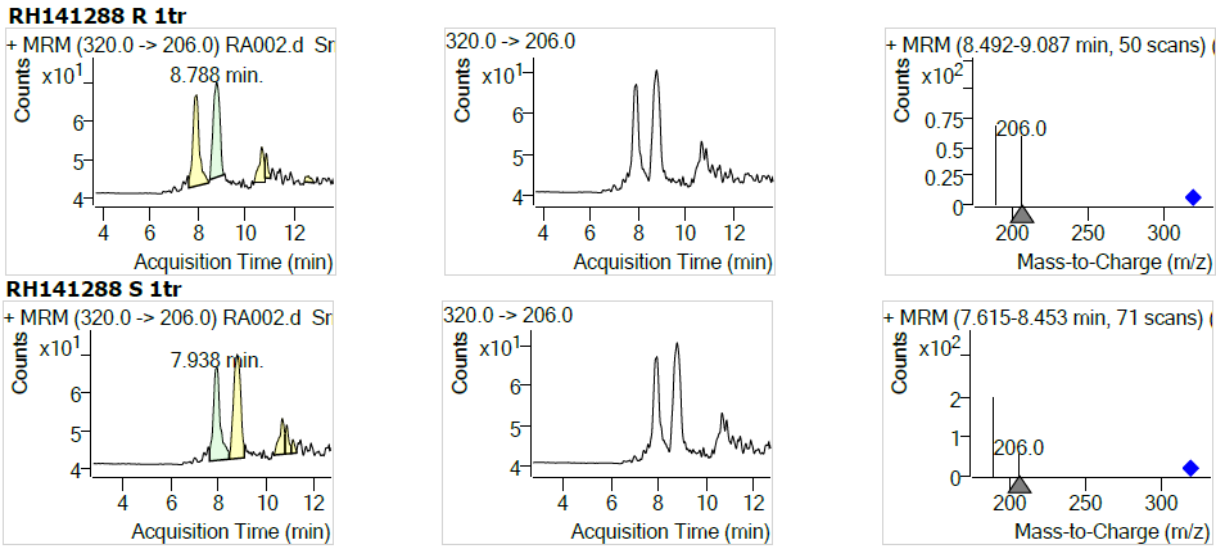
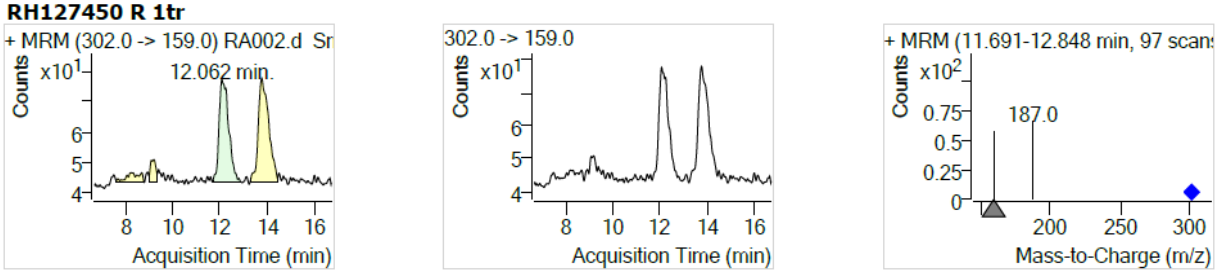


Figure A 31: Chromatogram of RH-141288 (R/S)



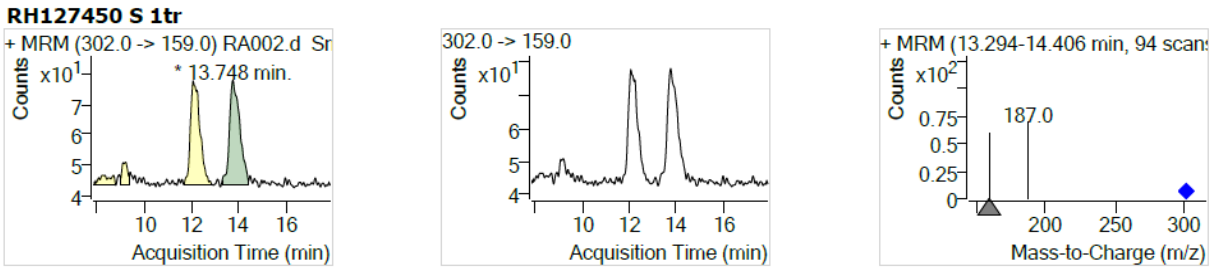


Figure A 32: Chromatogram of RH-127450 (R/S)

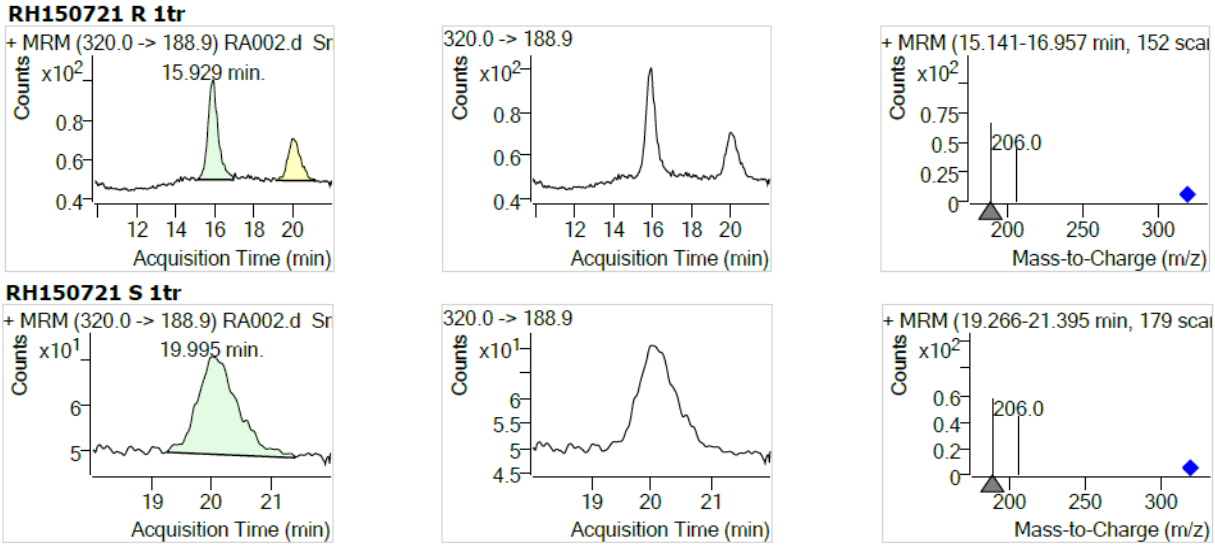


Figure A 33: Chromatogram of RH-150721 (R/S)

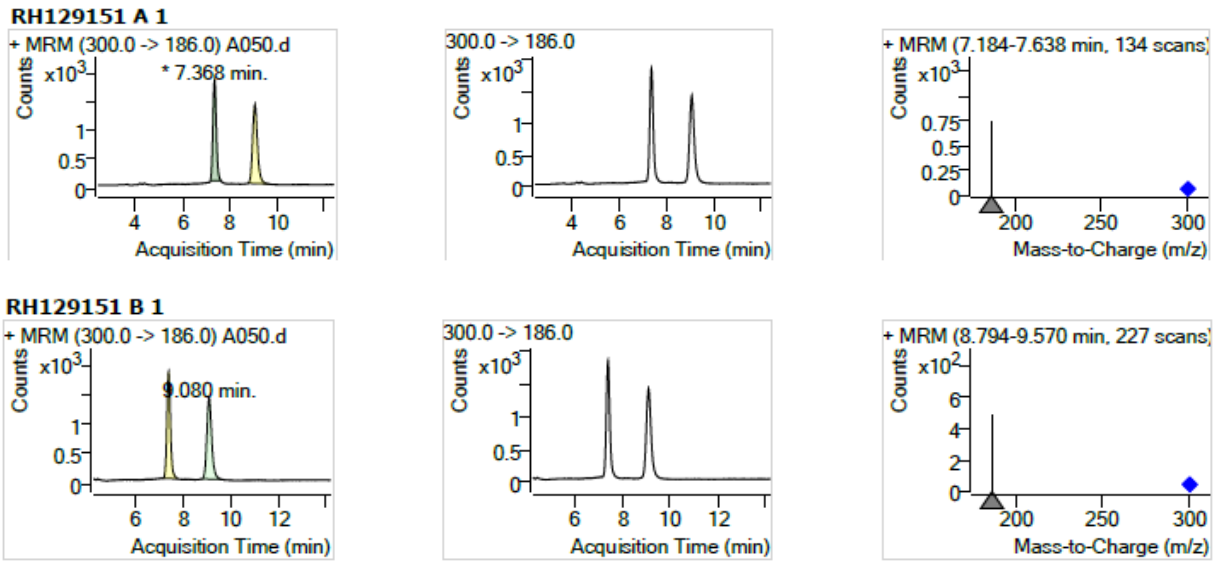


Figure A 34: Chromatogram of RH-129151 (A/B)

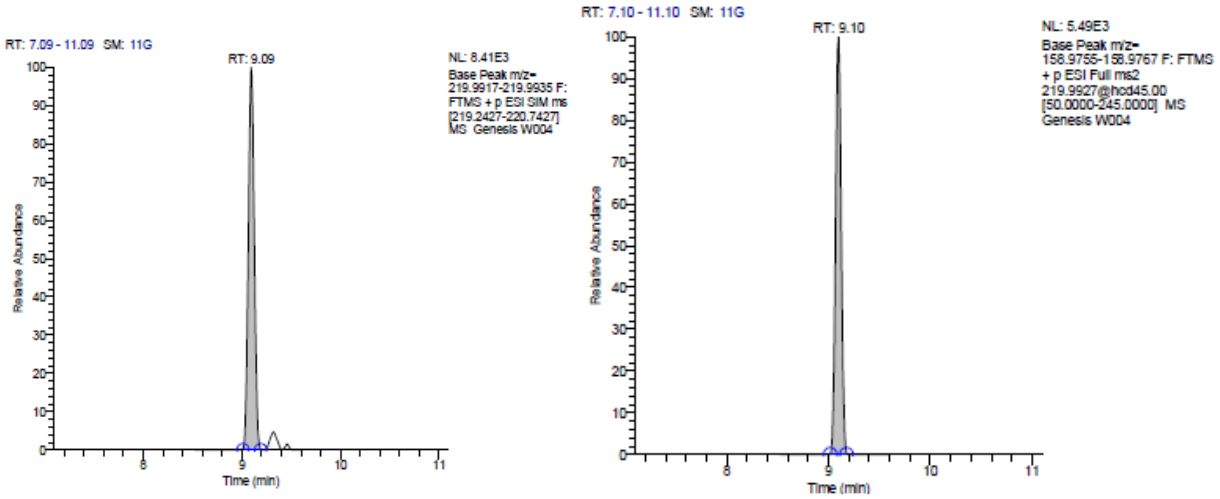


Figure A 35: Chromatogram of RH-149736

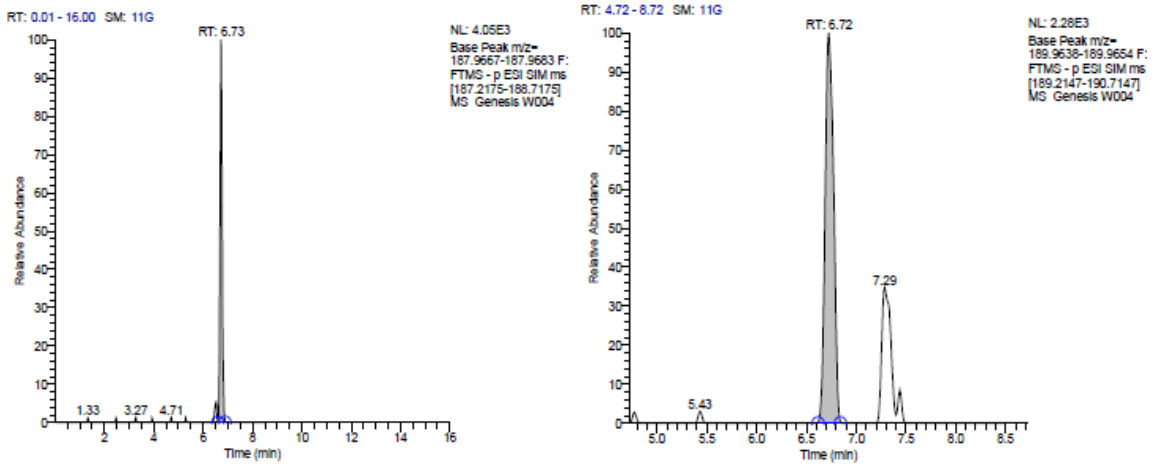


Figure A 36: Chromatogram of RH-149737

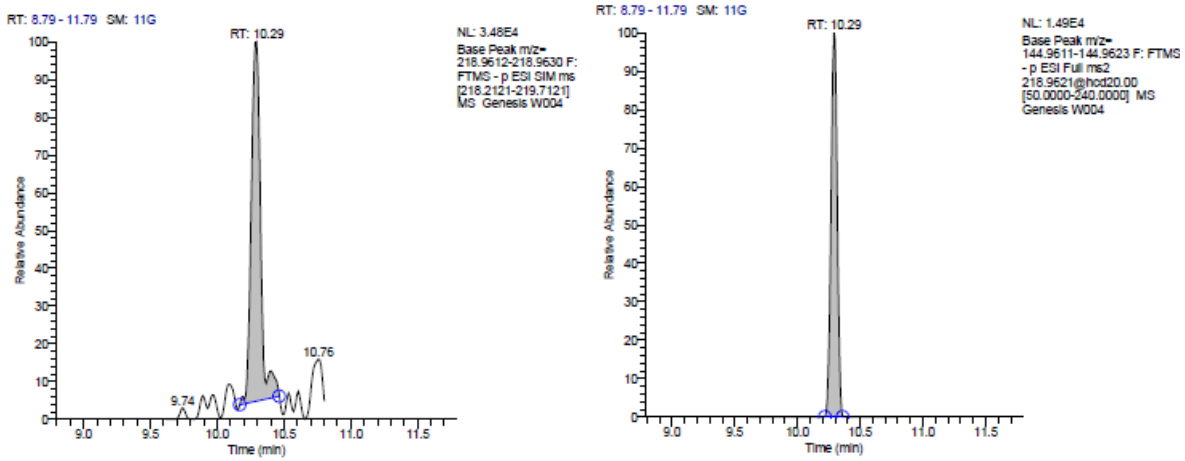


Figure A 37: Chromatogram of RH-141452

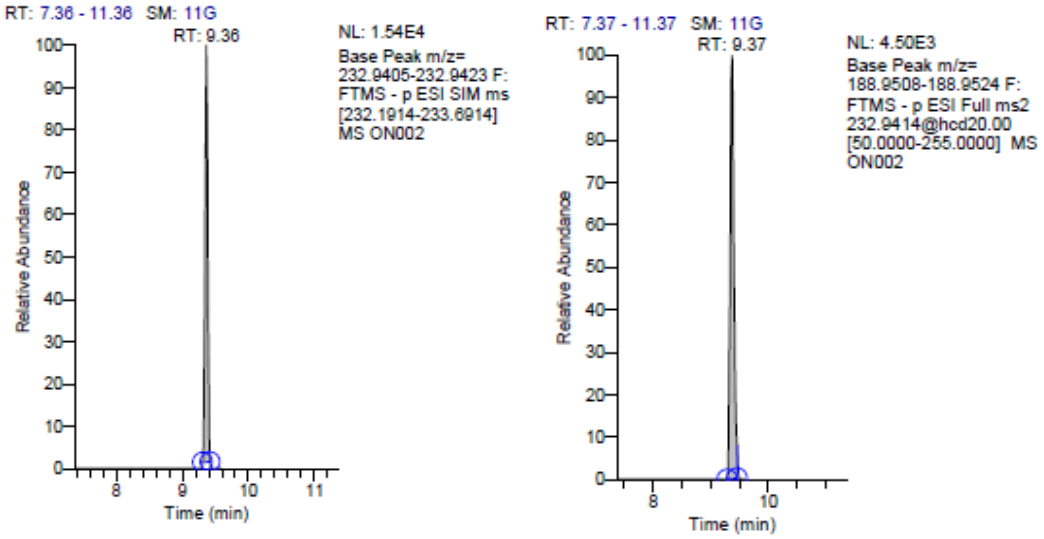


Figure A 38: Chromatogram of RH-141455

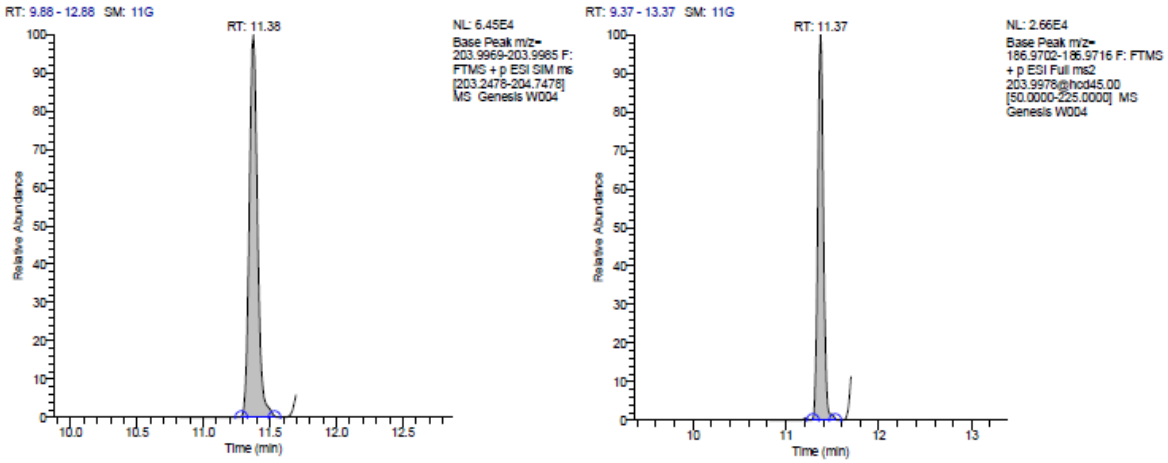


Figure A 39: Chromatogram of RH-139432

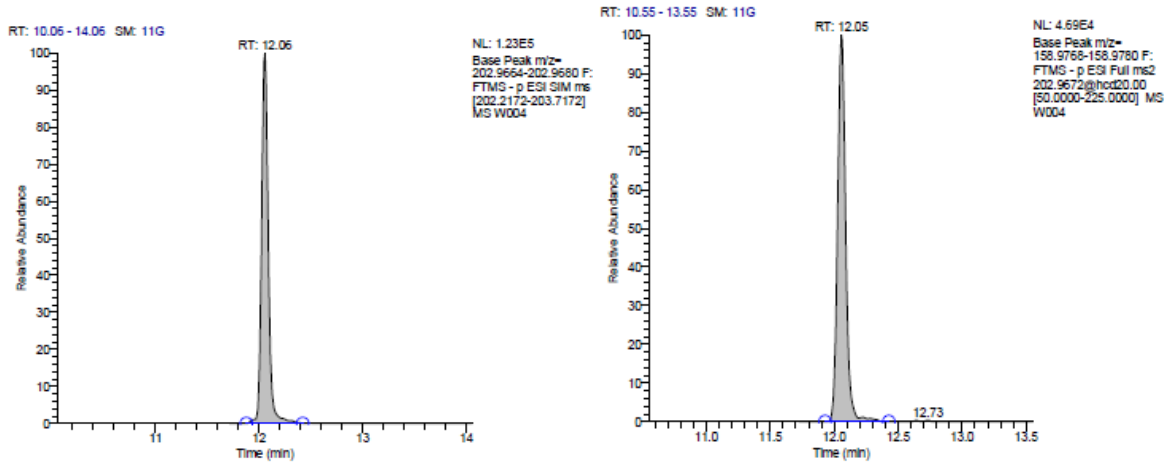


Figure A 40: Chromatogram of RH-24549

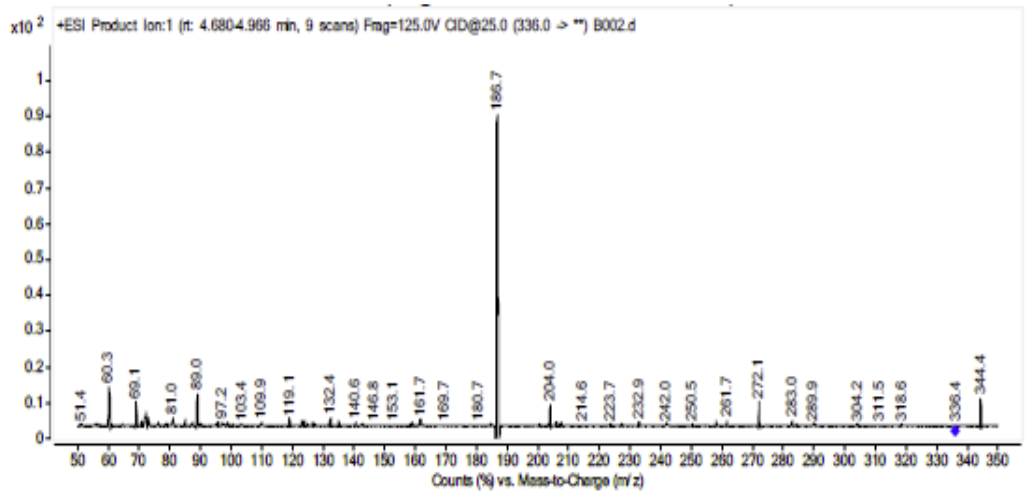


Figure A 41: Ion scan spectrum of zoxamide

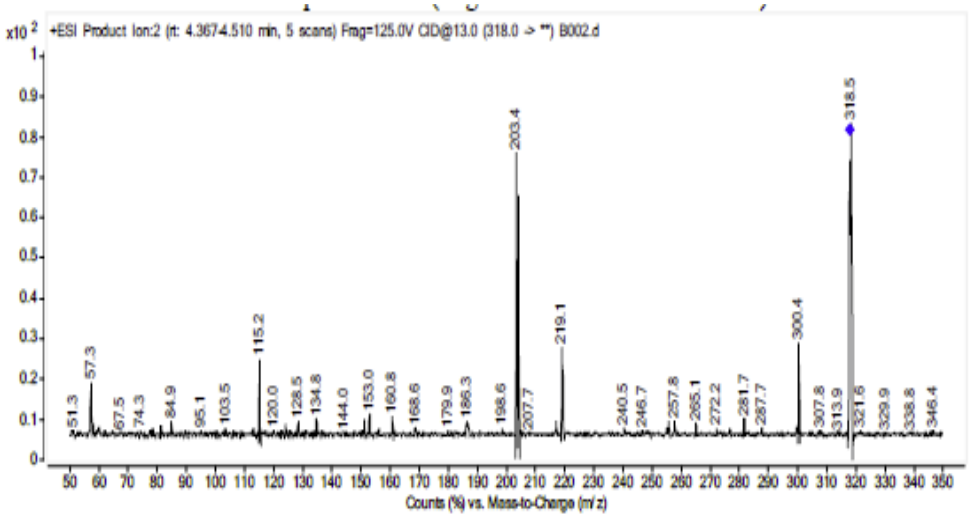


Figure A 42: Ion scan spectrum of RH-141288

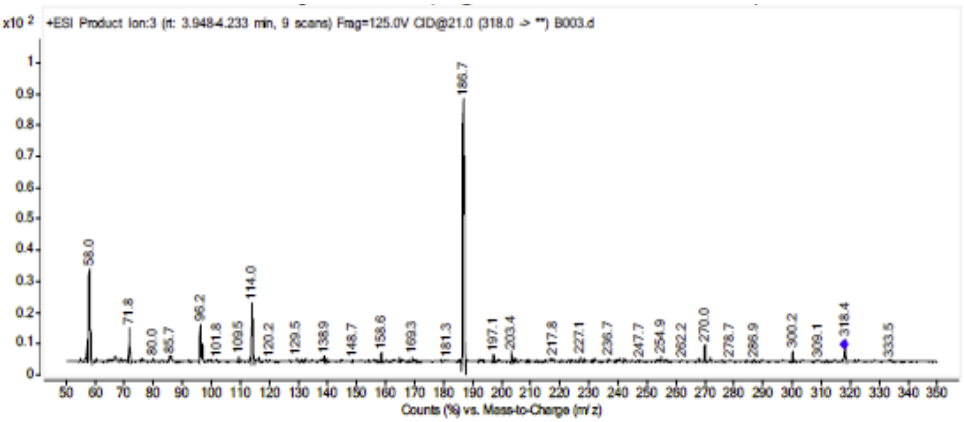


Figure A 43: Ion scan spectrum of RH-150721

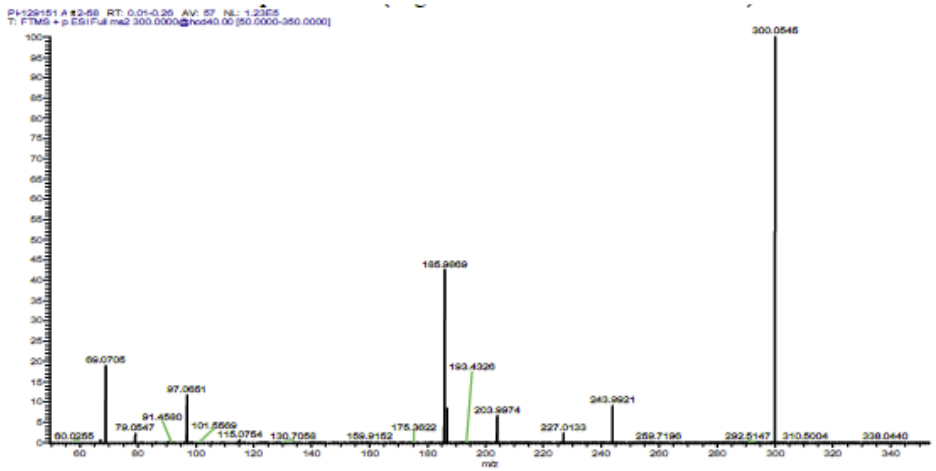


Figure A 44: Ion scan spectrum of RH-129151

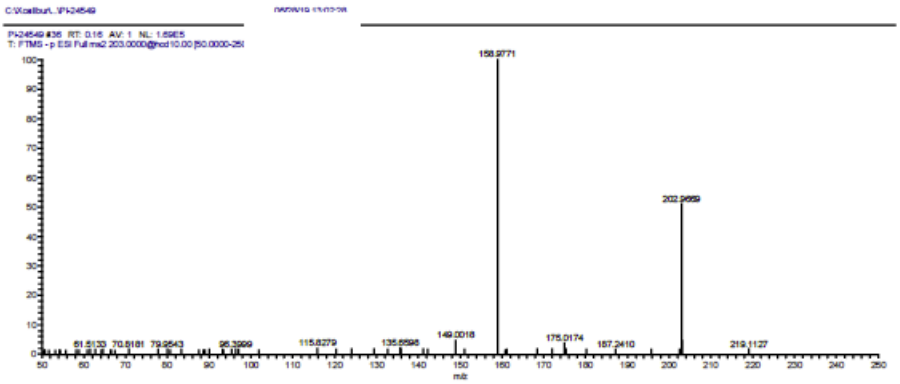


Figure A 45: Ion scan spectrum of RH-24549

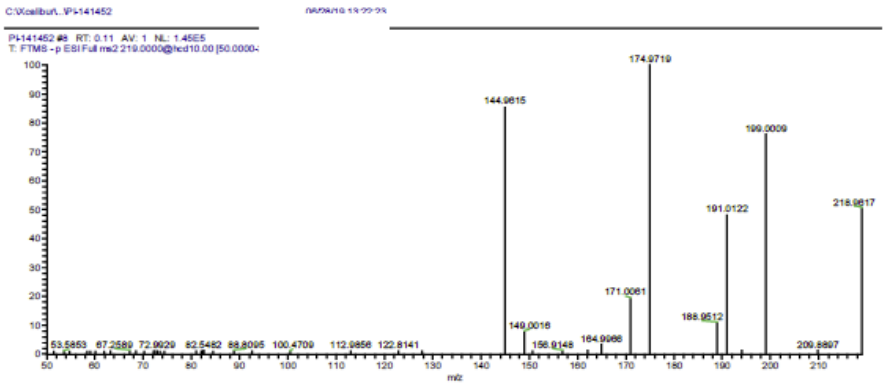


Figure A 46: Ion scan spectrum of RH-141452

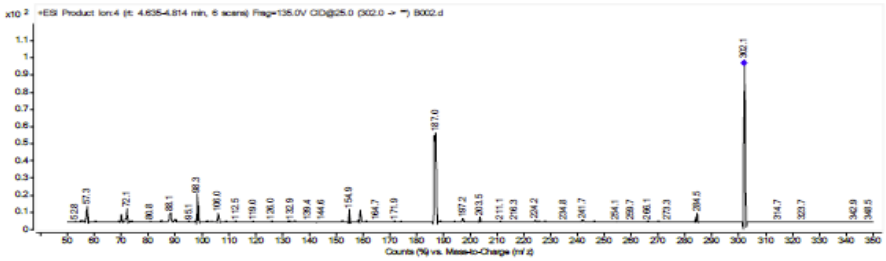


Figure A 47: Ion scan spectrum of RH-127450

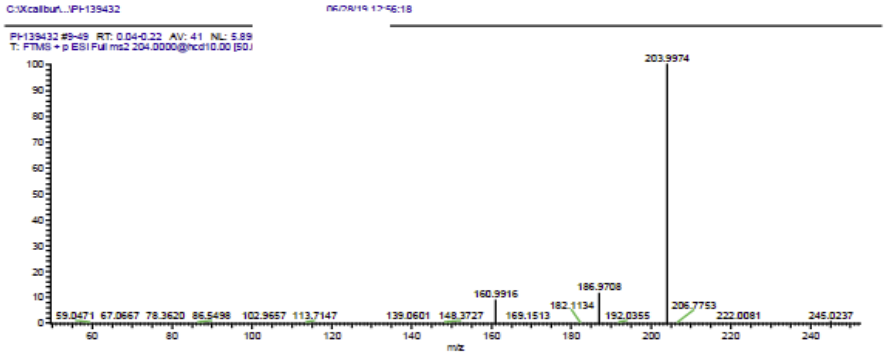


Figure A 48: Ion scan spectrum of RH-139432

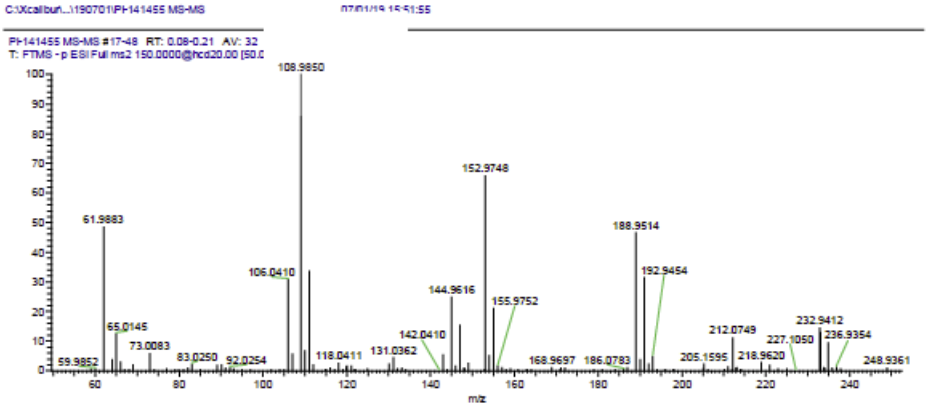


Figure A 49: Ion scan spectrum of RH-141455

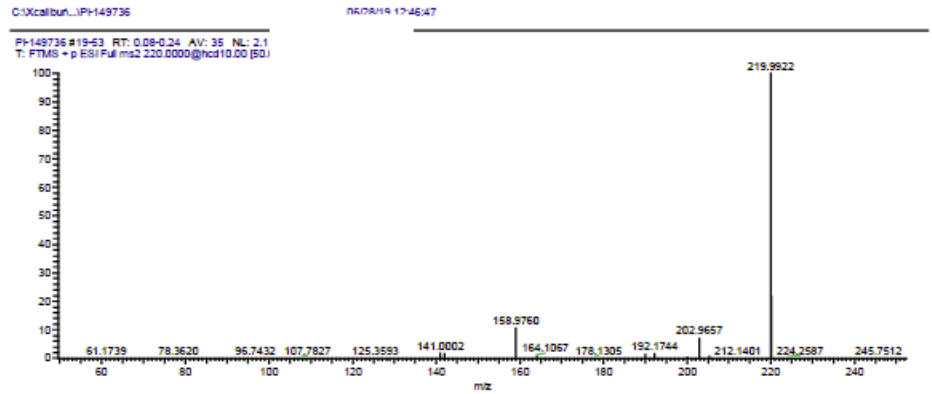


Figure A 50: Ion scan spectrum of RH-149736

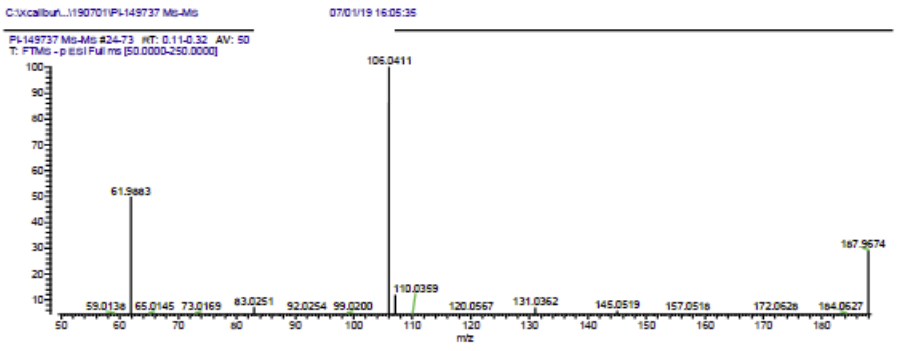


Figure A 51: Ion scan spectrum of RH-149737

Extraction efficiency

The extraction efficiency of the final analytical method has been studied in compliance with SANTE 2017/10632 rev. 3 by comparing the recoveries obtained applying the method under validation with the recoveries of the methods used in the plant metabolism studies performed with radiolabelled test item – and therefore allowing the bilancing of the analytes. A cross validation approach was applied.

Representative crop samples were sprayed with a mixture containing all the analytes. The sprayed samples were left 3 days at room temperature in daylight. After this period the samples were frozen, homogenised by a vegetable grinder, and analysed with the analytical method under validation. In parallel, aliquots of the same samples were extracted with the methods used in the plant metabolism studies, which are also summarised in the RAR zoxamide (2017). The results obtained with the 2 different extraction procedures were compared to assess the extraction efficiency of the method.

For all 4 matrices tested (grapes berries, potato tuber, tomato fruits and cucumber fruits) 3 mL of a water mixture containing all the analytes to be determined at a concentration of 100 µg/mL (each analyte) was applied. According to SANTE 2017/10632 rev. 3 the extraction efficiency was considered sufficiently proven if the residue extracted with the method under validation and the residue extracted with the method reported in the relevant plant metabolism study differs no more than 30%.

The following table summarises the results of extraction efficiency check:

Table A 127: Extraction efficiency for analytes from RAC samples - grape fruits

Analyte	Replicate	Analyte peak area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-Zoxamide	1	81852	89214	+2%
	2	91088	86621	
(S)-Zoxamide	1	72308	74843	-2%
	2	76504	70532	
(R)-RH-150721	1	154482	156253	-4%
	2	170255	156819	
(S)-RH-150721	1	250461	247878	-1%
	2	260499	258590	
RH-139432	1	3559481	2565170	-17%
	2	3383644	3222062	
RH-24549	1	4171856	3186616	-13%
	2	4136767	4048628	
RH-141452	1	1379681	1086517	-9%
	2	1373294	1408383	
RH-129151 (A)	1	23402162	20828250	-10%
	2	23665786	21303594	
RH-129151 (B)	1	22193362	20868087	-6%
	2	21979252	20692495	
(R)-RH-141288	1	12505	11221	-12%
	2	12071	10492	
(S)-RH-141288	1	5320	5026	-10%
	2	5438	4699	
RH-149736	1	522164	378331	-14%
	2	493359	493311	

Analyte	Replicate	Analyte peak area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-RH-127450	1	66474	70123	+3%
	2	67799	68275	
(S)-RH-127450	1	87867	97113	0%
	2	93774	85103	
RH-149737	1	262596	240245	+6%
	2	263265	315253	

Table A 128: Extraction efficiency for analytes from RAC samples - potato tubers

Analyte	Replicate	Analyte area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-Zoxamide	1	88075	91964	+5%
	2	89201	94891	
(S)-Zoxamide	1	62899	72442	+6%
	2	73640	71767	
(R)-RH-150721	1	249032	271447	+5%
	2	272127	278019	
(S)-RH-150721	1	293180	309124	+9%
	2	299064	336353	
RH-24549	1	9453938	9607449	-1%
	2	9355099	9044082	
RH-141452	1	4667107	4821012	-1%
	2	4592359	4382744	
RH-141455	1	2386000	2550540	+3%
	2	2365070	2363198	
RH-129151 (A)	1	23046998	21674663	-4%
	2	22223143	21890623	
RH-129151 (B)	1	21528424	20342993	-5%
	2	21282012	20276995	
(R)-RH-141288	1	53869	55259	-8%
	2	69748	58710	
(S)-RH-141288	1	53260	69109	+20%
	2	62614	70394	

Table A 129: Extraction efficiency for analytes from RAC samples - tomato fruits

Analyte	Replicate	Analyte area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-Zoxamide	1	83905	90994	+4%
	2	91147	90728	
(S)-Zoxamide	1	75763	91580	+15%
	2	84524	93109	

Analyte	Replicate	Analyte area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-RH-150721	1	169120	207012	+18%
	2	183471	210100	
(S)-RH-150721	1	284660	335145	+10%
	2	317201	329640	
RH-139432	1	2230571	2018713	-3%
	2	2082695	2148639	
RH-24549	1	2936002	2674100	-2%
	2	2643388	2772934	
RH-141452	1	1902036	1815960	-4%
	2	1911972	1860780	
RH-129151 (A)	1	21991802	21772526	0%
	2	21195613	21630008	
RH-129151 (B)	1	20469934	20107901	0%
	2	20018221	20357183	
(R)-RH-141288	1	6549	7695	+9%
	2	7005	7131	
(S)-RH-141288	1	5430	5841	+1%
	2	5895	5615	
RH-149736	1	610292	586978	-4%
	2	609946	580792	
(R)-RH-127450	1	78125	86430	+2%
	2	87494	83187	
(S)-RH-127450	1	103994	120885	+13%
	2	108706	118719	
RH-149737	1	365765	444948	+29%
	2	364025	494130	

Table A 130: Extraction efficiency for analytes from RAC samples - cucumber fruits

Analyte	Replicate	Analyte area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
(R)-Zoxamide	1	136364	116047	-8%
	2	124755	123961	
(S)-Zoxamide	1	135159	126455	+2%
	2	121201	134124	
(R)-RH-150721	1	392693	387673	+7%
	2	359808	419802	
(S)-RH-150721	1	402736	359426	-9%
	2	386068	354656	
RH-139432	1	2137497	2094957	-1%
	2	1962368	1965768	

Analyte	Replicate	Analyte area		Extraction efficiency % (mean value of 2 independent extractions)
		Metabolism study extraction method	Analytical method under validation	
RH-24549	1	3154266	3062499	-2%
	2	3096751	3049544	
RH-141452	1	1192494	1094435	-4%
	2	1122965	1130272	
RH-129151 (A)	1	20390772	20925150	+2%
	2	20445072	20856380	
RH-129151 (B)	1	18298852	19078967	+2%
	2	18797528	18858549	
(R)-RH-141288	1	8672	10480	+22%
	2	8970	11005	
(S)-RH-141288	1	38991	39812	+12%
	2	34786	42826	
RH-149736	1	324943	299121	-8%
	2	324075	296866	
(R)-RH-127450	1	130607	122719	0%
	2	119007	127396	
(R)-RH-127450	1	185545	169812	+1%
	2	158706	178419	
RH-149737	1	281996	258974	-8%
	2	291635	266754	

Conclusion

A highly specific HPLC-MS/MS method for the determination of zoxamide and its metabolites residues in grape, potato, tomato, cucumber, and onion raw agricultural and processed commodities has been validated according to SANCO/825/00 rev. 8.1 (2010) and SANCO/3029/99 rev. 4 (2000) at an LOQ of 0.01 mg/kg (for analytes with a chiral center this limit is referred to the sum of the 2 enantiomers; i.e. the LOQ for each enantiomer is 0.005 mg/kg). The extraction efficiency of the method has been demonstrated for different crop commodities (acidic, high starch content and watery matrix).

(Sala A. 2020)

A 2.1.2.1.3 Analytical method 3 – determination of zoxamide in honey

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The highly specific HPLC-MS/MS method was acceptably validated according to SANCO/3029/99 rev. 4 and SANCO/825/00 guidelines complies with SANTE/2020/12830, Rev.1 for determination of zoxamide in Phacelia honey after application of GWN-9790EU (a 240 g/L zoxamide SC formulation) at a worst-case use pattern of 3 x 180 g a.s./ha with a minimum interval of 7(+1) days under semi-field conditions (in field tunnels) with a limit of quantification (LOQ) of 0.01 mg/kg and a limit of detection of 0.003 mg/kg.*

Reference:	KCP 5.2 (a)/06
Report:	MAGNITUDE OF RESIDUES OF ZOXAMIDE IN PHACELIA (PHACELIA TANACETIFOLIA BENTH.) HONEY AFTER THREE APPLICATIONS OF GWN-9790EU UNDER SEMI-FIELD CONDITIONS IN NORTHERN AND SOUTHERN EUROPE, Poráczki, K., 2020, report No. 19 48 BTR 0003, Doc. No. 634-96001
Guideline(s):	OECD No. 506, Series on Testing and Assessment No. 72 and Series on Pesticides No. 39. ENV/JM/MONO(2007)17, SANCO/10684/2009, SANCO/825/00 rev. 8.1 (2010), SANTE/11813/2017 rev. 0, SANTE/11956/2016 rev. 9
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of the analytical phase of the study was the determination of residues of zoxamide (racemate) in honey after field applications of the test item GWN-9790EU to *Phacelia tanacetifolia* under semi-field conditions in Northern and Southern Europe.

The analysis of specimens was conducted by an in-house developed method based on the extraction method of the multi-residue method (QuEChERS method) of Anastassiades (2003) using high performance liquid chromatography (HPLC) on a chiral column and mass-spectrometric (MS-MS) detection at a limit of quantification of 0.01 mg/kg for Zoxamide (racemate) based on the method of Jooß (2013). This method is regarded as highly specific. Zoxamide (racemate) concentrations were determined as sum of both isomer peaks. The reference standards of the two single isomers of the active substance Zoxamide will be used to distinct between the (R)- and the (S)-isomer peaks.

For sample preparation, to 3 g of samples (honey) each 15 mL of water and acetonitrile were added prior to extraction on a Fastprep instrument (3 cycles, 20 s at speed 5 m/s, 15 s pause). Afterwards, about 3.5 g salt mixture (4:1:1:1 magnesium sulfate: sodium chloride: trisodium citrate dihydrate: disodium hydrogen citrate sesquihydrate) were added and the Fastprep extraction was repeated (3 cycles, 20 s at speed 5 m/s, 15 s pause). All samples were centrifuged at 5000 rpm for 5 min. The resulting acetonitrile phases were diluted with water (and blank extract, if necessary) and injected into the HPLC-system. The two enantiomers of zoxamide were separated on a chiral column by high performance liquid chromatography (HPLC). Detection was carried out by tandem mass-spectrometry, monitoring two mass transitions for each

enantiomer (m/z: 336 → 187 (quantifier) and 336 → 159 (qualifier)). The analysis was performed with external, matrix-matched standards.

After extraction of the honey samples from the combs, the respective specimen containers were stored at under deep-frozen conditions (≤ -18 °C).

Equipment

Instrument: A Shimadzu LC-20 system with a LC-8040 triple quadrupole mass spectrometric detector

Column: Phenomenex Lux Cellulose-3, 3 µm, 150*2.0 mm

Column temp.: 35°C

Mobile phase: A: water containing 0.1% formic acid and 5 mM ammonia formate
B: methanol containing 0.1% formic acid and 5 mM ammonia formate

Time (min)	A %	B %
0.00	40	60
7.00	0	100
9.00	0	100
9.01	40	60
11.00	40	60

Flow rate: 0.3 mL/min

Injection volume: 10 µL

Run time: 11.00 min, 2 min re-equilibration time

Detector type: ESI positive, Multiple Reaction Monitoring (MRM),
Zoxamide: m/z: 336→187 (quantifier), 336→159 (qualifier)

Retention times: 6.8 min for R-zoxamide; 7.6 min for S-zoxamide

Results and discussions

Recovery findings

Summaries of the results are presented in the following table.

Table A 131: Recovery results for zoxamide (racemate)

Validation level	n	Concentration [mg/kg]	Recovery [mg/kg]	Mean recovery [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (quantifier)						
LOQ	5	0.010	0.0092, 0.0091, 0.0091,0.0085, 0.0082	0.0088	88	5.18
100x LOQ	5	1.002	0.923, 0.931, 0.922, 0.944, 0.920	0.928	93	1.08
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (qualifier)						
LOQ	5	0.010	0.0091, 0.0090, 0.0091, 0.0084, 0.0086	0.0088	88	4.08
100x LOQ	5	1.002	0.945, 0.956, 0.940, 0.971, 0.946	0.951	95	1.29
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Table A 132: Recovery results for R-zoxamide

Validation level	n	Concentration [mg/kg]	Recovery [mg/kg]	Mean recovery [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (quantifier)						
LOQ	5	0.005	0.0037, 0.0036, 0.0036, 0.0034, 0.0032	0.0035	70	5.43
100x LOQ	5	0.499	0.373, 0.382, 0.378, 0.389, 0.375	0.379	76	1.58
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (qualifier)						
LOQ	5	0.010	0.0036, 0.0036, 0.0036, 0.0033, 0.0034	0.0035	70	3.34
100x LOQ	5	1.002	0.376, 0.382, 0.374, 0.387, 0.376	0.379	76	1.45
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Table A 133: Recovery results for S-zoxamide

Validation level	n	Concentration [mg/kg]	Recovery [mg/kg]	Mean recovery [mg/kg]	Mean recovery [%]	RSD [%]
m/z 336→187 (quantifier)						
LOQ	5	0.005	0.0051, 0.0050, 0.0050, 0.0045, 0.0044	0.0048	95	6.69
100x LOQ	5	0.503	0.454, 0.453, 0.448, 0.458, 0.449	0.453	90	0.88
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-
m/z 336→159 (qualifier)						
LOQ	5	0.010	0.0051, 0.0050, 0.0051, 0.0044, 0.0046	0.0048	96	6.91
100x LOQ	5	1.002	0.452, 0.456, 0.450, 0.464, 0.453	0.455	90	1.23
Blank	2	0.000	<30% LOQ	< 30% LOQ	-	-

Accuracy and precision / repeatability

The validity of the analytical method was proven by analysis of spiked validation samples (high and low spiked validation concentration), five replicates each, and two validation blank samples. The recovery values were in the range of 70 – 110% with relative standard

deviations (RSDs) ≤ 20%, which demonstrates acceptable accuracy and precision of the method.

Linearity

The calibration functions were linear in the range of 0.282 to 7.05 µg/L for zoxamide (racemate), in the range of 0.147 to 3.68 µg/L of R-zoxamide and in the range of 0.147 to 3.67 µg/L of S-zoxamide. 1/x weighting was applied and a correlation coefficient of > 0.99 was obtained.

Limit of quantification

The limit of quantification (LOQ) was 0.010 mg/kg of zoxamide (racemate) in honey, corresponding to 1.00 µg/L in diluted sample extracts and 0.005 mg/kg of the single enantiomers of zoxamide in honey (R-zoxamide and S-zoxamide, corresponding to 0.50 µg/L in diluted sample extracts).

Limit of detection

The limit of detection (LOD) was 0.003 mg/kg of zoxamide (racemate) and 0.0015 mg/kg of the single enantiomers of zoxamide (R-zoxamide and S-zoxamide) in honey specimens.

Matrix effects

For all analyses, matrix-matched calibration standards were used in this analytical phase.

Specificity

The method is regarded as highly specific and is able to separate the two enantiomers of zoxamide (R-zoxamide and S-zoxamide). It uses liquid chromatography (with a chiral column) with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions per analyte for quantification and/or confirmation. In addition, interfering peaks in control samples, which eluted at the same retention times, were either non-detectable or amounted to less than 30% of the limit of quantification (LOQ).

Storage Stability of sample extracts

Stability of sample extracts was tested in BioChem project No.: 18 35 CRB 0040, analytical phase to BioChem project No.: 18 48 BFB 0001. Sample extracts were stable for a period of 11 days at 4-10°C. The maximum storage period of sample extracts in this analytical phase was 6 days

Storage Stability of frozen samples

The maximum freezer storage period for honey samples of the field part was 83 days. The stability of honey samples in the freezer was demonstrated by recovery experiments over a storage period of at least 85 days.

Table A 134: Characteristics of the analytical method for the determination of zoxamide in honey

	Zoxamide (racemate)	**R-zoxamide**	**S-zoxamide**
Specificity	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)	HPLC with MS/MS detection, monitoring two ion mass transitions for quantification and/or qualification; characteristic retention time of the analyte; no interfering peaks (blank value < 30% LOQ)
Calibration (type, number of data points)	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 22687.6 x + 8715.03, r^2=0.99986	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 313719 x + 10908.6, r^2=0.99884	7-point calibration with external standard/ solvent calibration The calibration was linear and weighted 1/c Calibration curve equation: y = 261849 x + 39336.4, r^2=0.99884
Calibration range	0.282-7.05 µg/L in analytical samples or 0.003 to 1.41 mg/kg in honey specimens	0.147-3.68 µg/L in analytical samples or 0.0015 to 0.736 mg/kg in honey specimens	0.147-3.67 µg/L in analytical samples or 0.0015 to 0.733 mg/kg in honey specimens
Assessment of matrix effects is presented	Matrix-matched calibration standards.	Matrix-matched calibration standards.	Matrix-matched calibration standards.
Limit of determination/ quantification	LOQ: 0.010 mg/kg, corresponding to 1.00 µg/L in the analytical sample. LOD: 0.003 mg/kg, corresponding to 0.282 µg/L in the analytical sample	LOQ: 0.005 mg/kg, corresponding to 0.500 µg/L in the analytical sample. LOD: 0.0015 mg/kg, corresponding to 0.147 µg/L in the analytical sample	LOQ: 0.005 mg/kg, corresponding to 0.500 µg/L in the analytical sample. LOD: 0.0015 mg/kg, corresponding to 0.147 µg/L in the analytical sample

The following figures show typical chromatograms.

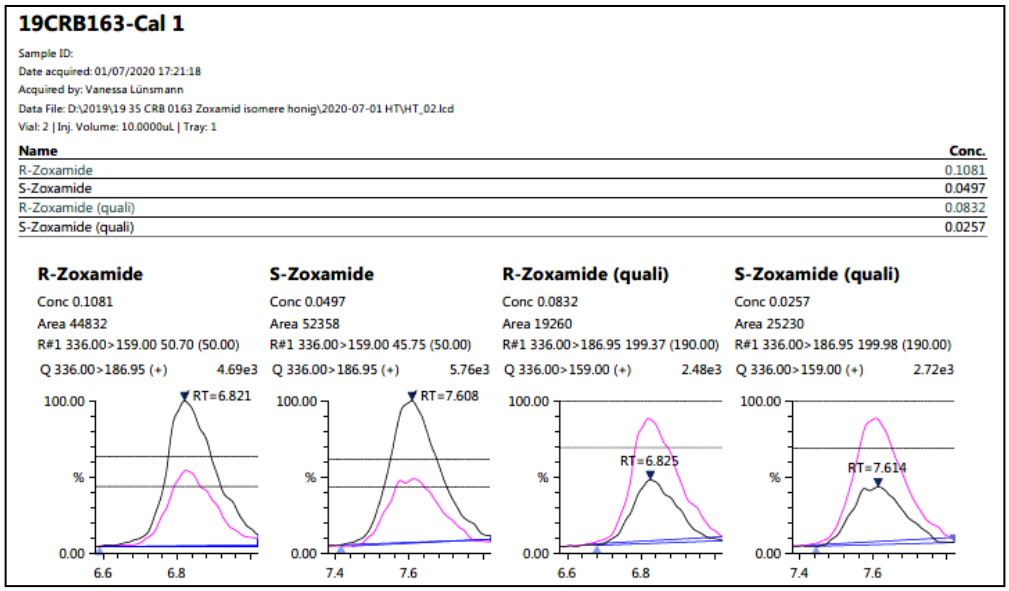


Figure A 52: Chromatogram of calibration standard zoxamide (racemate)

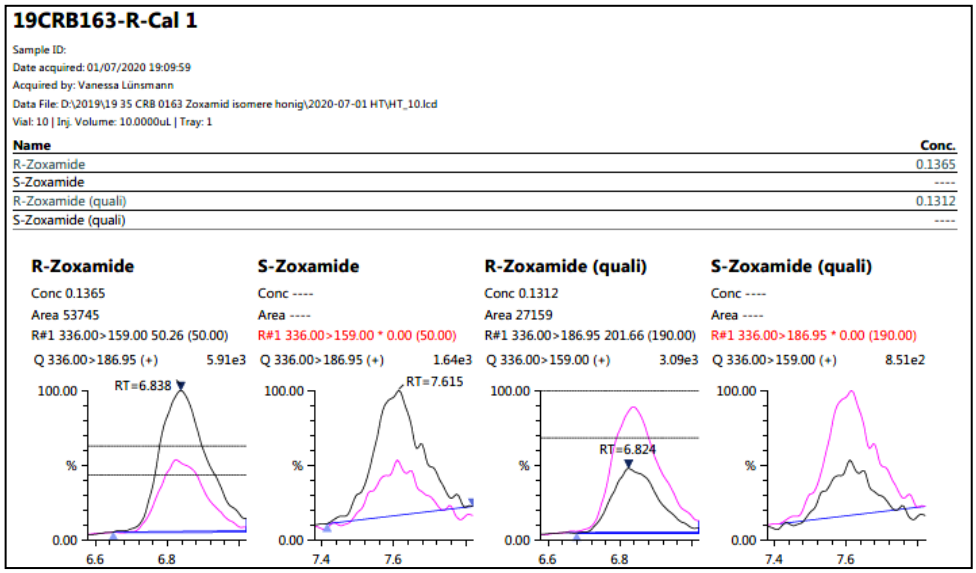


Figure A 53: Chromatogram of calibration standard R-Zoxamide

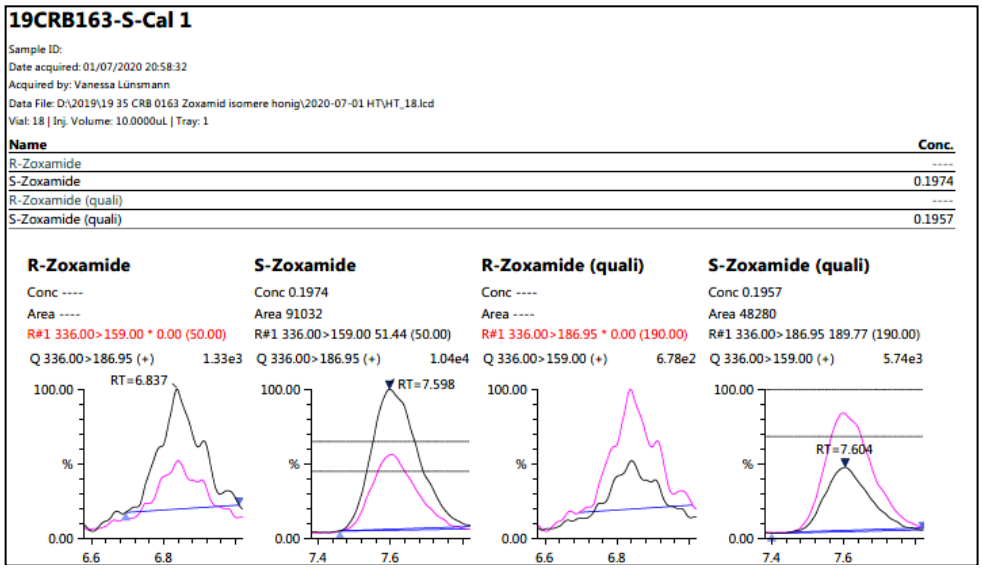


Figure A 54: Chromatogram of calibration standard S-Zoxamide

Conclusion

The method for the determination of zoxamide in honey was fully validated according to SANCO/3029/99 rev. 4 (2000) and SANCO/825/00 rev. 8.1 (2010) with a limit of quantification (LOQ) of 0.01 mg/kg and a limit of detection (LOD) of 0.003 mg/kg. The method is also regarded in-line with SANTE/2020/12830 rev. 1 (2021).

Independent laboratory validation for honey

The method for honey summarised above was based on the multi-residue method Quechers. The Quechers method has also been used for the analysis of plants (please refer to the studies evaluated during EU review for Zoxamide renewal). The ILV for the Quechers method in plants (Richter, S. (2014), Report no. P3114G, EU agreed method) can therefore be considered as ILV for honey. Furthermore, according to the DIN EN 15662 Norm, the validity of the Quechers method has been confirmed for Zoxamide in samples of low water and high sugar content, to which honey belongs. Thus, the Quechers method has been independently validated for Zoxamide in honey.

A 2.1.2.1.3.1

A 2.1.2.1.3.2 Analytical method 4

This active substance related study has already been provided to the RMS Latvia. Thus, the summary of the study is only presented for completeness sake. The study is only indicated in the list of data submitted or referred to by the applicant and relied on.

Comments of RMS: ***Latvia***	*The following conclusion of RMS Latvia was taken from Circa and originated from part B section 5 core assessment for products GF-1045, GF-1057, GWN-9790EU, GWN-9823, GWN-9963, GOW F911 WG, GOW F113 finalised in September 2023 as updated set of zoxamide data after AIR submitted by XXXX. and its affiliates in May 2021:* *The method based on QuEChERS method is fit for monitoring of zoxamide isomer residues in 4 different matrices: potato tubers (water containing matrix), potato flakes (dry matrix), potato chips (fat containing matrix) and pickled silver-skin*

	onions (acidic matrix). The method has been sufficiently validated according to the SANCO/825/00 rev. 8.1 guideline compliant with SANTE/2020/12830, Rev.1 (2021).

Reference:	KCP 5.2 (a)/07
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF R/S-ISOMERS OF ZOXAMIDE AND METABOLITES RH-141452 AND RH-141455 IN 4 DIFFERENT MATRICES: POTATO TUBERS (WATER CONTAINING MATRIX), POTATO FLAKES (DRY MATRIX), POTATO CHIPS (FAT CONTAINING MATRIX) AND PICKLED SILVERSKIN ONIONS (ACIDIC MATRIX), Witte, A., 2020, report No. 18G10186-01-VMPL, Doc. No. 432-011
Guideline(s):	SANCO/825/00 rev. 8.1 (2010) ENV/JM/MONO (2017)17 series 72/39
Deviations:	No
GLP:	Yes
Acceptability:	Yes

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

The method of Witte (2020) can be seen as primary and/or ILV enforcement/monitoring method for the determination of zoxamide (R/S and sum) and RH-141452 and RH-141455 in all matrices (high water content, acidic, high oil content and dry) – especially for the determination of RH-141452 and RH-141455 in raw and processed commodities of root and tuber crops.

Component(s) analysed: RH-141455 and RH-141452				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Potato tubers Potato flakes Potato chips Pickled silverskin onions	Primary Confirmatory	Potato tuber: 0.01 mg/kg Potato flakes Potato chips: 0.05 mg/kg	LC-MS/MS	Weber & Giesau, 2013 Report no. S12-03951 EU agreed method (RAR 2017)
	Primary / ILV Confirmatory	0.01 mg/kg	LC-MS/MS	Witte, 2020 Report no. 18G10186-01-VMPL
Component(s) analysed: R- and S-zoxamide and sum				
Potato tubers Potato flakes Potato chips Pickled silverskin onions	Primary / ILV Confirmatory	0.005 mg/kg	LC-MS/MS	Witte, 2020 Report no. 18G10186-01-VMPL

Materials and methods

The data presented in this report demonstrate that the method used in analogy to the multiresidue method QuEChERS (EN15662) permits the determination of residues of zoxamide isomers in matrices potato chips, potato flakes, pickled silverskin onions and potato tubers. Furthermore, the method used for zoxamide metabolites RH-141452 and RH-141455 was proven to be suitable for all four tested matrices for RH-141452

and RH-141455, but unsuitable for zoxamide (racemate) itself. The methods were validated in different plant tissues according to the guideline SANCO/825/00 rev. 8.1 (2010), i.e. in potato chips, potato flakes, pickled silverskin onions and potato tubers. The methods were proven to be specific, accurate and precise and a good repeatability and recovery was found in all four matrix groups for all analytes except for zoxamide (racemate).

For zoxamide isomers, specimens were weighed into 50 mL single-use centrifuge tubes. Recovery samples were fortified at this step. 10 mL acetonitrile was added and the samples were homogenised for at least 2 min using a vortex mixer. Thereafter, QuEChERS EN15662 salt-mixture (1 g sodium citrate, 0.5 g sodium hydrogencitrate sequihydrate, 4 g magnesium sulphate, 1 g sodium chloride) was added, thoroughly shaken and mixed again on a vortex mixer for at least 1 min. The samples were centrifuged at 4000 min-1 for at least 5 minutes. An aliquot of 1 mL of the supernatant was transferred into a tube prepared with 25 mg PSA (primary-secondary amino phase) and 150 mg anhydrous magnesia sulphate and mixed on a Vortex mixer for 1 min. The extract was filtered through a single–use syringe filter (0.45 µm) into an autosampler vial (1.8 mL). 50 µL of this sample extract were then diluted with 950 µL acetonitrile/water (20:80, v/v) + 0.1 % formic acid and analysed via HPLC-MS/MS.

For zoxamide (racemate) and metabolites, specimens were weighed into 150 mL screw capped glass bottles. Before sample preparation, specimens of potato chips and potato flakes were soaked with 16 g of water. After addition of 50 mL of extraction solution (methanol/0.01 N sodium hydroxide solution (7:3, v/v)), specimens were homogenised for 2 min using a high-speed homogeniser and centrifuged at 3800 rpm for 10 min. The supernatant was transferred into a 100 mL volumetric flask. Once again, 30 mL of methanol/0.01 N sodium hydroxide solution (7:3, v/v) was added into the 150 mL screw capped glass bottle and the extract was homogenised for 1 min and centrifuged again. The supernatant was transferred into the same 100 mL volumetric flask. The combined extract solutions in the 100 mL volumetric flask were filled up to 100 mL (VEX) with the methanol/0.01 N sodium hydroxide solution (7:3, v/v). Next, an aliquot of 10 mL (VAL1) of the extract was mixed with 25 mL of 0.01 N sodium hydroxide solution. The obtained solution contained 20 % methanol.

Control specimens of each matrix were analysed in duplicate and fortified specimens were analysed in quintuple. Since two characteristic mass transitions were used to monitor each analyte, the method achieves a high level of specificity and no additional confirmation was necessary.

Equipment for zoxamide isomers

HPLC-MS/MS:	Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP
Column:	Phenomenex LUX® 5 µm Cellulose-3 150mm × 4.6 mm, 5.0 µm (Part No. 00F-4493-E0)
Mobile phase:	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid

Time (min)	A (%)	B (%)
0.00	40	60
0.10	40	60
8.00	25	75
8.10	40	60
12.00	40	60

Flow rate:	1 mL/min
Column temp.:	40°C
Injection volume:	100 µL
Retention time:	(R) – Zoxamide ~ 3.8 min (S) – Zoxamide ~ 4.3 min
Quantification:	Peak areas of the fragment ions, matrix-matched external standards
Ion mode:	Zoxamide: m/z 336 → 187; m/z 338 → 189

Equipment for zoxamide (racemate) and metabolites

HPLC-MS/MS: Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP

Column: Phenomenex SynergieTM 4 µm Polar – RP 80A 150 mm × 4.6 mm, 4.0 µm (Part No. 00F-4493-E0)

Mobile phase: A: water + 0.05% acetic acid
B: methanol + 0.05% acetic acid

Time (min)	A (%)	B (%)
0.00	80	20
2.00	40	60
5.00	20	80
9.50	20	80
9.60	80	20
15.10	80	20

Flow rate: 1 mL/min

Column temp.: 40°C

Injection volume: 100 µL

Retention time: Zoxamide (racemate) ~ 8.9 min
RH-141452 ~ 5.7 min
RH-141455 ~ 3.5 min

Quantification: Peak areas of the fragment ions, matrix-matched external standards

Ion mode: **Zoxamide:** m/z 336 → 187; m/z 338 → 189
RH-141452:
m/z 219 → 175; m/z 221 → 147 for matrices potato flaked, potato tubers and pickled silver skin onions;
m/z 221 → 147; m/z 221 → 177 for matrix potato chips
RH-141455:
m/z 233 → 189; m/z 235 → 191 for matrices potato flaked, potato tubers and pickled silver skin onions;
m/z 235 → 191; m/z 233 → 109 for matrix potato chips

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

Results and discussions

Recovery findings

Summaries of the results are presented in the following table.

Table A 135: Results of accuracy, recovery precision and repeatability ((R)-zoxamide)

Matrix	Fortification Level [mg/kg]	Recoveries			No. of Analyses	Overall recovery	
		Single Values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
(R) - Zoxamide SRM 336 → 187 (quantification)							
Potato tubers	0.005	108, 114, 120, 102, 110	111	6.1	5	105	9.3
	0.05	96, 92, 109, 90, 108	99	9.0	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato tubers	0.005	110, 114, 118, 102, 110	111	5.4	5	106	9.2
	0.05	97, 92, 113, 90, 110	100	10.5	5		

(R) - Zoxamide SRM 336 → 187 (quantification)							
Potato flakes	0.005	100, 100, 94, 92, 98	97	3.8	5	102	6.1
	0.05	101, 110, 109, 105, 108	107	3.4	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato flakes	0.005	102, 98, 96, 94, 102	98	3.6	5	103	5.6
	0.05	103, 111, 110, 104, 108	107	3.3	5		
(R) - Zoxamide SRM 336 → 187 (quantification)							
Potato chips	0.005	100, 94, 100, 102, 100	99	3.1	5	105	6.4
	0.05	111, 109, 117, 108, 106	110	3.8	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Potato chips	0.005	98, 94, 100, 102, 100	99	3.1	5	105	6.9
	0.05	111, 111, 117, 108, 110	111	3.0	5		
(R) - Zoxamide SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.005	104, 110, 116, 118, 108	111	5.2	5	103	9.8
	0.05	87, 100, 93, 96, 98	95	5.3	5		
(R) - Zoxamide SRM 338 → 189 (confirmation)							
Pickled silverskin onions	0.005	102, 108, 118, 118, 108	111	6.3	5	103	10.2
	0.05	86, 98, 93, 96, 98	94	5.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 136: Results of accuracy, recovery precision and repeatability ((S)-zoxamide)

Matrix	Fortification Level [mg/kg]	Recoveries			No. of Analyses	Overall recovery	
		Single Values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato tubers	0.005	112, 112, 128, 102, 110	113	8.4	5	109	8.3
	0.05	107, 94, 113, 102, 112	106	7.4	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato tubers	0.005	112, 114, 128, 104, 110	114	7.8	5	110	8.4
	0.05	108, 93, 115, 102, 111	106	8.1	5		
(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato flakes	0.005	100, 104, 100, 94, 106	101	4.6	5	104	6.2
	0.05	98, 110, 116, 108, 105	107	6.2	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato flakes	0.005	100, 104, 104, 96, 106	102	3.9	5	104	5.5
	0.05	95, 108, 113, 108, 108	106	6.3	5		

(S) - Zoxamide SRM 336 → 187 (quantification)							
Potato chips	0.005	92, 90, 92, 90, 92	91	1.2	5	95	5.4
	0.05	97, 105, 102, 97, 93	99	4.8	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Potato chips	0.005	94, 86, 98, 90, 94	92	4.9	5	96	5.9
	0.05	101, 105, 101, 97, 93	99	4.6	5		
(S) - Zoxamide SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.005	116, 114, 114, 114, 124	116	3.7	5	107	10.4
	0.05	91, 97, 103, 97, 96	97	4.4	5		
(S) - Zoxamide SRM 338 → 189 (confirmation)							
Pickled silverskin onions	0.005	114, 116, 118, 118, 128	119	4.5	5	108	11.3
	0.05	94, 99, 104, 96, 94	97	4.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 137: Results of accuracy, recovery precision and repeatability (zoxamide (racemate))

Matrix	Fortification Level [mg/kg]	Recoveries			No. of Analyses	Overall recovery	
		Single Values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato tubers	0.01	-	-	-	5	-	-
	0.10	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato tubers	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato flakes	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato flakes	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Potato chips	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Potato chips	0.01	-	-	-	5	-	-

	0.1	-	-	-	5		
Zoxamide (racemate) SRM 336 → 187 (quantification)							
Pickled silverskin onions	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		
Zoxamide (racemate) SRM 338 → 189 (confirmation)							
Pickled silverskin onions	0.01	-	-	-	5	-	-
	0.1	-	-	-	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 138: Results of accuracy, recovery precision and repeatability (RH-141452)

Matrix	Fortification Level [mg/kg]	Recoveries			No. of Analyses	Overall recovery	
		Single Values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
RH-141452 SRM 219 → 175 (quantification)							
Potato tubers	0.01	99, 100, 104, 93, 106	100	5.0	5	97	6.1
	0.1	98, 88, 92, 90, 99	93	5.2	5		
RH-141452 SRM 221 → 147 (confirmation)							
Potato tubers	0.01	103, 104, 108, 101, 101	103	2.8	5	99	6.9
	0.1	97, 90, 92, 87, 102	94	6.3	5		
RH-141452 SRM 219 → 175 (quantification)							
Potato flakes	0.01	114, 101, 92, 103, 115	105	9.2	5	100	8.6
	0.1	101, 97, 98, 91, 91	96	4.6	5		
RH-141452 SRM 221 → 147 (confirmation)							
Potato flakes	0.01	115, 110, 91, 104, 116	107	9.6	5	101	9.6
	0.1	100, 94, 99, 89, 95	95	4.6	5		
RH-141452 SRM 221 → 147 (quantification)							
Potato chips	0.01	111, 116, 95, 102, 113	107	8.1	5	108	6.3
	0.1	113, 99, 108, 108, 110	108	4.8	5		
RH-141452 SRM 221 → 177 (confirmation)							
Potato chips	0.01	114, 113, 97, 112, 106	108	6.6	5	108	6.0
	0.1	113, 97, 109, 112, 111	108	6.1	5		
RH-141452 SRM 219 → 175 (quantification)							
Pickled silverskin onions	0.01	102, 97, 95, 103, 102	100	3.6	5	99	4.4
	0.1	105, 91, 96, 96, 98	97	5.2	5		
RH-141452 SRM 221 → 147 (confirmation)							

Pickled silverskin onions	0.01	94, 90, 93, 101, 101	96	5.2	5	96	5.5
	0.1	106, 89, 97, 97, 95	97	6.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Table A 139: Results of accuracy, recovery precision and repeatability (RH-141455)

Matrix	Fortification Level [mg/kg]	Recoveries			No. of Analyses	Overall recovery	
		Single Values [%]	Mean [%]	RSD [%]		Mean [%]	RSD [%]
RH-141455 SRM 235 → 191 (quantification)							
Potato tubers	0.01	101, 100, 90, 91, 105	97	6.8	5	96	6.2
	0.1	98, 90, 96, 88, 102	95	6.1	5		
RH-141455 SRM 223 → 109 (confirmation)							
Potato tubers	0.01	105, 101, 106, 105, 104	104	1.8	5	101	5.2
	0.1	100, 93, 100, 91, 105	98	5.8	5		
RH-141455 SRM 233 → 189 (quantification)							
Potato flakes	0.01	104, 94, 97, 85, 89	94	7.8	5	92	8.0
	0.1	93, 92, 101, 79, 89	91	8.7	5		
RH-141455 SRM 235 → 191 (confirmation)							
Potato flakes	0.01	103, 102, 96, 93, 100	99	4.2	5	95	6.8
	0.1	92, 93, 101, 82, 91	92	7.3	5		
RH-141455 SRM 223 → 189 (quantification)							
Potato chips	0.01	109, 110, 103, 105, 115	108	4.3	5	106	5.5
	0.1	108, 95, 111, 106, 100	104	6.2	5		
RH-141455 SRM 235 → 191 (confirmation)							
Potato chips	0.01	128, 104, 112, 120, 116	116	7.7	5	110	8.6
	0.1	113, 96, 106, 105, 102	104	6.0	5		
RH-141455 SRM 233 → 189 (quantification)							
Pickled silverskin onions	0.01	102, 92, 104, 101, 102	100	4.7	5	96	6.3
	0.1	96, 86, 91, 92, 93	92	4.0	5		
RH-141455 SRM 235 → 191 (confirmation)							
Pickled silverskin onions	0.01	102, 94, 99, 104, 100	100	3.8	5	96	5.7
	0.1	97, 86, 92, 93, 92	92	4.3	5		

RSD = relative standard deviation; SRM: single reaction monitoring

Mean recovery values within a range of 70 - 120% obtained by HPLC-MS/MS for all matrices comply with the standard acceptance criteria of guideline SANCO/825/00 rev. 8.1 (2010).

Excluded from this is zoxamide (racemate), for which the extraction method suitable for RH-141452 and RH-141455 was not applicable.

Accuracy and precision / repeatability

Fortification experiments for zoxamide isomers were performed in five-fold at the limit of quantification (0.005 mg/kg) and at 10-fold of the limit of quantification (0.05 mg/kg). For zoxamide (racemate) and its metabolites RH-141452 and RH-141455, fortifications were performed at the 0.01 mg/kg (LOQ) and 0.1 mg/kg (10-fold LOQ).

Mean recovery values obtained by HPLC-MS/MS for all matrices comply with the standard acceptance criteria of guideline SANCO/825/00 rev. 8.1 (2010), which require that the mean recoveries should be within the range of 70 - 120%. Therefore, it can be concluded that the methods are applicable on all matrices under investigation using HPLC with MS/MS detection. Excluded from this is zoxamide (racemate), which was not suitable for the extraction method like its metabolites RH-141452 and RH-141455.

Moreover, all corresponding relative standard deviations are below 20% as required by the guideline SANCO/825/00 rev. 8.1 (2010). This indicates that the method demonstrates good precision and repeatability for the different plant tissues at the validated levels. The repeatability of the method was assessed on the basis of the obtained relative standard deviations for each fortification level

Linearity

The linearity of the detector response for (R)- and (S)-zoxamide was confirmed by injecting eight matrix matched standard solutions (same solutions as described in 6.4) covering the working range of 0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions (eight-point calibration) and 0.0125 µg/L to 10 µg/L for matrices potato flakes and potato chips (seven-point calibration) with correlation coefficients of $r \geq 0.998$.

The linearity of the detector response for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 was confirmed by injecting seven matrix matched standard solutions (same solutions as described in 6.4) covering the working range of 0.3 µg/L to 20 µg/L for all matrices with correlation coefficients of $r \geq 0.997$.

The lower margin of the linearity test was below 30% of the LOQ and the upper margin was higher by at least 20 % as the 10-fold LOQ. These margins cover the minimum range as required by SANCO/825/00 rev. 8.1 (2010).

Limit of quantification

The limit of quantification (LOQ) was defined as the lowest fortification level at 0.005 mg/kg for zoxamide Isomers and 0.01 mg/kg for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 with mean recoveries ranging from 70% to 120% at a relative standard deviation (RSD) of ≤ 20% and blanks not exceeding 30% of the LOQ. These criteria were fulfilled for all matrices and analytes - except for zoxamide (racemate), for which the tested method was deemed unsuitable.

Limit of detection

The limit of detection (LOD) was defined as 30% of the limit of quantification as required by SANCO/825/00 rev. 8.1 (2010) for residues in control samples (i.e. 0.0015 mg/kg and 0.003 mg/kg). Residues in the untreated samples used for recovery experiments were below 30% of the LOQ, below the limit of detection (LOD) to be exact.

Matrix effects

The matrix effect was tested at concentrations levels of 10 µg/L for all matrices. Standard solutions were injected in pure solvent (double injection) against matrix-matched standard solutions.

A considerable matrix effect of > 20 % for (S)–zoxamide was observed in potato tubers and pickled silverskin onions. No significant matrix effects (above 20 %) could be observed for (R)–zoxamide in any matrix. Nevertheless, matrix matched standards were used for all matrices.

For zoxamide (racemate), significant matrix effects of > 20 % were observed for all matrices except for pickled silverskin onions. Metabolites RH-141452 and RH-141455 showed significant matrix effects of > 20 % for all matrices.

Specificity

Analysis of control specimens (in duplicate) of each matrix with HPLC-MS/MS using two characteristic mass transitions yielded no residues of zoxamide (racemate) or its metabolites RH-141452 and RH-141455 above 30 % of the LOQ, indicating that no significant interferences were present.

Zoxamide parent compound with two characteristic mass transitions: m/z = 336 → 187 (quantification) and 338 → 189 (confirmation) for all matrices.
RH-141452: 219 → 175 and 221 → 147 for matrices potato flakes, potato tubers and pickled silverskin onions.
RH-141452: 221 → 147 and 221 → 177 for matrix potato chips.
RH-141455: 233 → 189 and 235 → 191 for matrices potato flakes, potato chips and pickled silverskin onions.
RH-141455: 235 → 191 and 233 → 109 for matrix potato tubers.

Stability of sample extracts

The stability of zoxamide isomers as well as zoxamide (racemate) and its metabolites RH-141452 and RH-141455 in final extracts was assessed by measuring matrix-matched calibration standards of each matrix stored refrigerated (≤ 8 °C) against freshly prepared matrix-matched calibration standards. Matrix-matched calibration standards were prepared in the final dilution of control samples. Stability tests for all analytes in final extracts were performed at a level of 10µg/L. The results demonstrate that no significant deviation (> 20 %) between stored and freshly prepared matrix-matched standards was observed for zoxamide (racemate) and its metabolites RH-141452 and RH-141455 after storage in a refrigerator (≤ 8 °C) for 8 (potato tubers), 7 (pickled silverskin onions), 8 (potato flakes) and 6 days (potato chips). Furthermore, no significant deviation (> 20 %) between stored and freshly prepared matrix-matched standards was observed for zoxamide isomers during 6 days of storage for all matrices.

The stability of the working solutions SM-Z1 and SM-RH1 (concentration 1 mg/L used for preparation of recoveries and standards; stored at ≤ 8 °C) was tested for 86 days. For analysis, stored and freshly prepared working solutions were diluted with acetonitrile (for zoxamide isomers) or methanol (for zoxamide (racemate) and its metabolites RH-141452 and RH-141455) to receive 100 µg/L standard solutions. Each standard was injected twice. The results demonstrate that no significant degradation (> 20 %) of zoxamide and its metabolites occurred in the working solution during storage.

Table A 140: Characteristics for the analytical method used for validation of zoxamide isomers, zoxamide (racemate) and metabolites residues in plant matrices

	(R)- and (S)-zoxamide	**Zoxamide (racemate), RH-141452 and RH-141455**
Specificity	HPLC-MS/MS is regarded highly specific. Mass spectrum is provided. Blank value above 30 % LOQ	HPLC-MS/MS is regarded highly specific. Mass spectrum is provided. Blank value above 30 % LOQ

Calibration (type, number of data points)	Standard solution and matrix matched calibration.
	Calibration range 0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions (eight-point calibration) and 0.0125 µg/L to 10 µg/L for matrices potato flakes and potato chips (seven-point calibration).
	Correlation coefficient r^2 was > 0.998 for each individual matrix.
	Individual calibration data and calibration line equation presented in the study report.
	y = 1.11277e6 x, r = 0.99996
		Standard solution and matrix matched calibration.
		Calibration range 0.3 µg/L to 20 µg/L for all matrices (seven-point calibration).
		Correlation coefficient r^2 was > 0.997 for each individual matrix.
		Individual calibration data and calibration line equation presented in the study report.
		y = 3.21286e5 x, r = 0.9992 (zoxamide racemat);
		y = 5.62462e5 x, r = 0.99958 (RH-141452);

		y = 2.66876e5 x, r = 0.99991
Calibration range	0.05 µg/L to 10 µg/L for matrices potato tubers and pickled silverskin onions and 0.0125 µg/L to 10 µg/L for matrices potato flakes and potato chips	0.3 µg/L to 20 µg/L for all matrices
Assessment of matrix effects is presented	yes	yes
Limit of determination/quantification	LOQ: 0.005 mg/kg of each zoxamide isomer LOD: 30 % of the LOQ (*i.e.* 0.0015 mg/kg)	LOQ: 0.01 mg/kg for each analyte LOD: 30 % of the LOQ (*i.e.* 0.003 mg/kg)

The following figures show representative mass spectra.

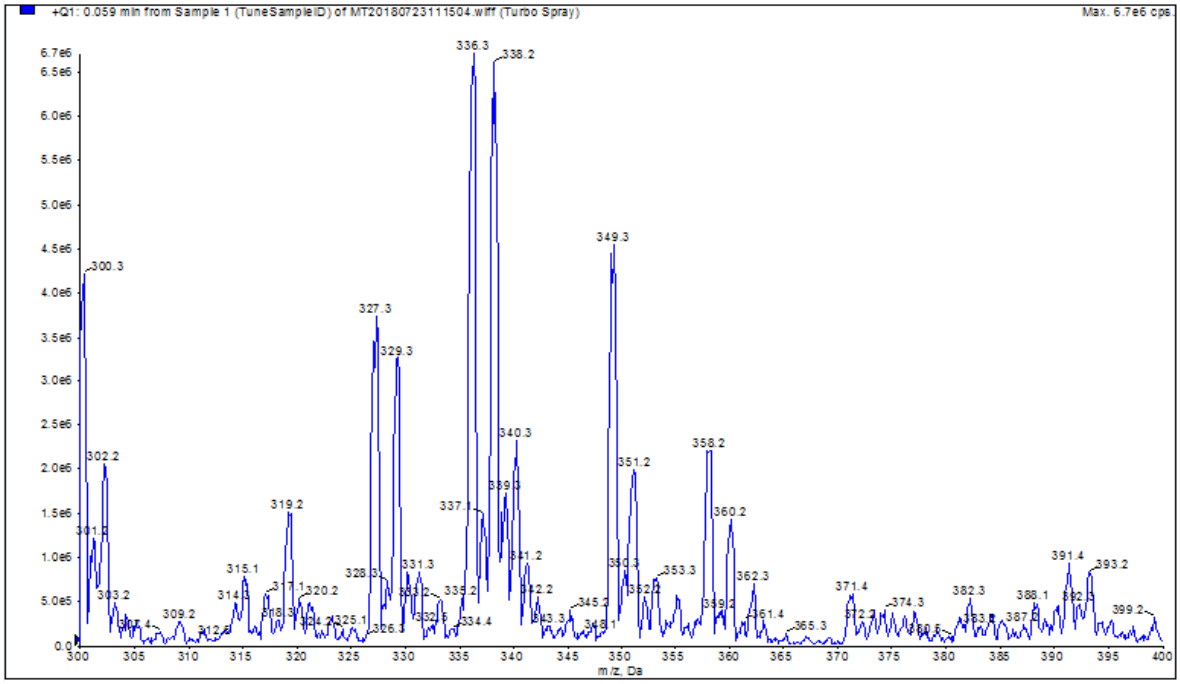


Figure A 55: Mass spectrum of zoxamide

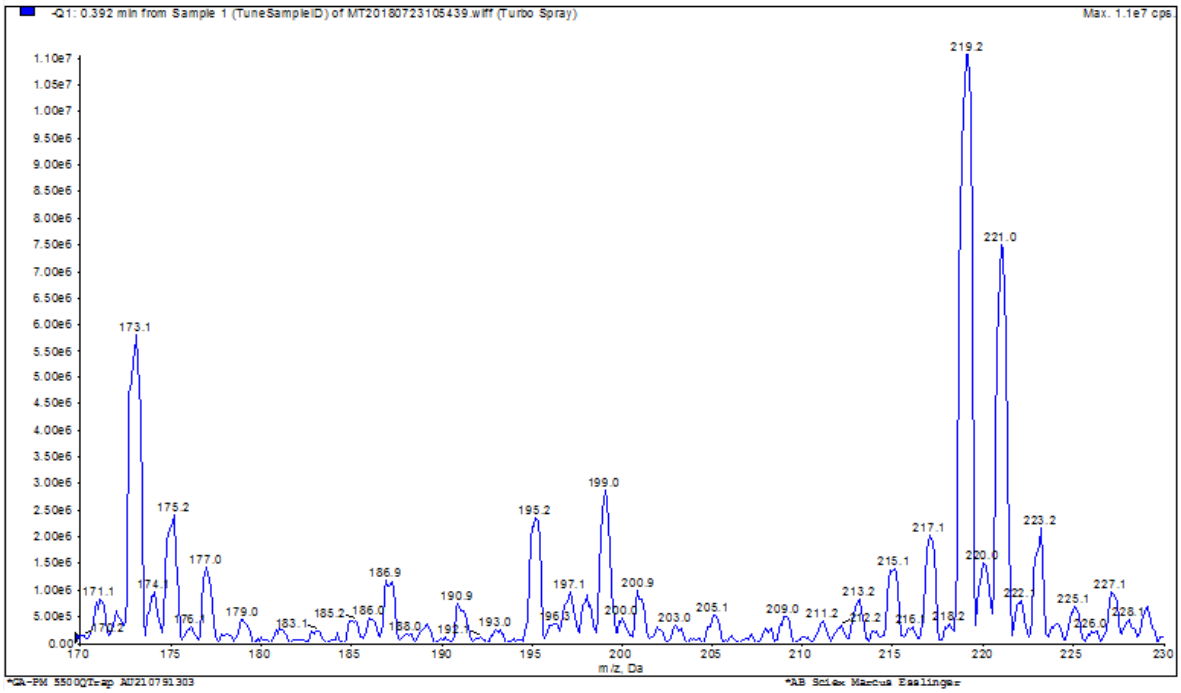


Figure A 56: Mass spectrum of RH-141452

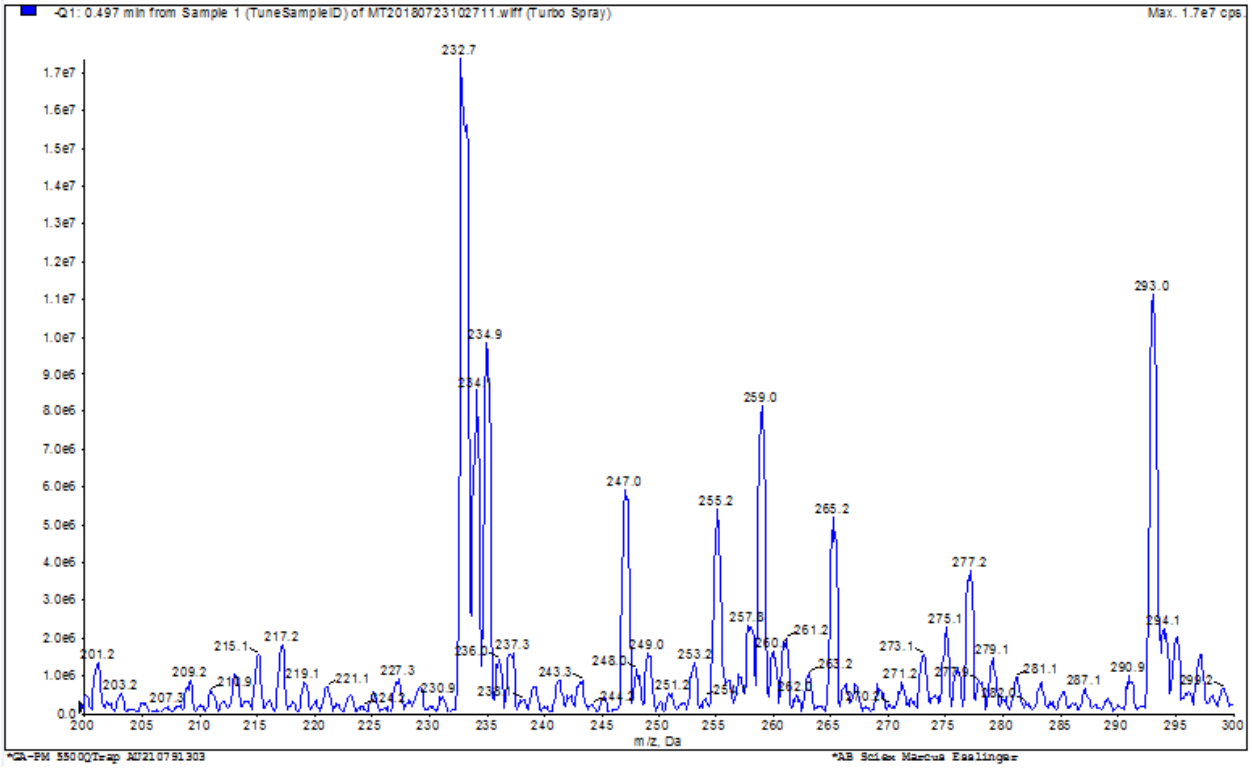


Figure A 57: Mass spectrum of RH-141455

Extraction efficiency

The extraction procedure used in this study was according to the QuEChERS method (validated for zoxamide in study No P 3114 G (RICHTER, S., PTRL Europe 2014)). This method was checked for its extraction efficiency in study AS362 (Hein, W., RLP AgroScience GmbH, Germany, 2014) in pea immature whole plant and dry peas. Therefore, the extraction efficiency was considered to be proven for dry and water containing plant material.

Conclusion

The applicability of a method (in analogy to the multiresidue method QuEChERS (EN 15662)) for analysis of residues of zoxamide isomers in different plant tissue matrices was tested, i.e. potato chips, potato flakes, pickled silverskin onions and potato tubers. The specimen extracts were analysed using liquid chromatography with mass selective detection (HPLC-MS/MS). The method was validated successfully according to SANCO/825/00 rev.8.1 (2010).

The method was proven to be specific, accurate and precise and good repeatability and recovery was found in all matrices. Therefore, this QuEChERS based method can be used for monitoring of zoxamide isomer residues in all tested matrix groups.

The applicability of the method for analysis of residues of zoxamide metabolites RH-141452 and RH-141455 was tested in the same matrices. The specimen extracts were also analysed using liquid chromatography with mass selective detection (HPLC-MS/MS). The method was validated successfully according to SANCO/825/00 rev.8.1 (2010) for metabolites RH-141452 and RH-141455, but deemed unsuitable for analysis of zoxamide itself.

The method was proven to be specific, accurate and precise and good repeatability and recovery was found in all matrices. Therefore, the method can be used for monitoring of zoxamide metabolites RH-141452 and RH-141455 residues in all tested matrix groups.

A 2.1.2.1.3.3 Confirmatory method (if required)

Confirmatory method was validated in the primary method validation, see above.

A 2.1.2.1.3.4 Extraction efficiency

Extraction efficiency has been demonstrated in the study report No. BPL-STUDY-18-000085, Doc. No. 432-009 which was submitted with the dRR to RMS Latvia. The study summary is included above for completeness. The new post-registration methods in apple and grape as well as the pre-registration method for Zoxamide in potato (report No. GLP-STUDY-21-50, Doc. No. 432-016) described above use the same extraction solvent as in this study which had been demonstrated to have sufficient extraction efficiency.

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

Not relevant for this submission. 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:	The validation has been accepted. The analytical method was based on determination using a LC- MS/MS. The animal matrices were extracted using a QuEChERS based method. The validation parameters were consistent with the requirements.

Reference:	KCP 5.2 (d)/01
Report:	VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTIFICATION OF ZOXAMIDE IN BODY FLUIDS AND TISSUE, Longhi, D., 2022, report No. LBN-0002-2022, Doc. No. 433-001
Guideline(s):	SANTE/2020/12830 rev. 1 (2021), ENV/JM/MONO(2007)17
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples of bovine meat were extracted with acetonitrile after addition of water. A Quechers salt mixture (MgSO4, NaCl, trisodium citrate dehydrate, disodium hydrogen citrate sesquihydrate) was added, and the sample was centrifuged. The final extract was analysed by HPLC-MS/MS.
Samples of bovine urine and plasma were extracted with acetonitrile. A Quechers salt mixture (MgSO4, NaCl, trisodium citrate dehydrate, disodium hydrogen citrate sesquihydrate) was added, and the sample was centrifuged. The final extract was analysed by HPLC-MS/MS.

Chromatographic conditions

System	HPLC-MS/MS
Column	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Mobile phase	Mobile phase A: LC-MS grade water with 0.1 % formic acid Mobile phase B: LC-MS grade acetonitrile with 0.1 % formic acid
Monitored ions	336.0 > 186.9 and 336.0 > 159.0
Retention time	2.2 min

Results and discussions

The mean recovery at each fortification level as well as the overall mean recovery were in the range of 70 – 110% with the relative standard deviation (RSD) below 20%. There were no significant interferences in the analysis of blank samples.

Table A 141: Recovery results from method validation of Zoxamide using the analytical method

Matrix	Analyte	Fortification level (mg/kg for tissues; mg/L for fluids) (n = x)	Mean recovery (%)	RSD (%)	Comments
Bovine meat	Zoxamide 336 > 186.9	0.01 (n = 5)	85.8	2.7	-
		0.1 (n = 5)	89.4	3.2	-
		Overall (n= 10)	87.6	3.5	-

Matrix	Analyte	Fortification level (mg/kg for tissues; mg/L for fluids) (n = x)	Mean recovery (%)	RSD (%)	Comments
Bovine meat	Zoxamide 336 > 159.0	0.01 (n = 5)	84.4	3.2	-
		0.1 (n = 5)	89.3	3.3	-
		Overall (n= 10)	86.8	4.3	-
Bovine plasma	Zoxamide 336 > 186.9	0.01 (n = 5)	93.6	2.7	-
		0.1 (n = 5)	97.5	1.5	-
		Overall (n= 10)	95.6	3.0	-
Bovine plasma	Zoxamide 336 > 159.0	0.01 (n = 5)	92.5	3.4	-
		0.1 (n = 5)	97.0	1.8	-
		Overall (n= 10)	94.8	3.5	-
Bovine urine	Zoxamide 336 > 186.9	0.01 (n = 5)	89.5	3.4	-
		0.1 (n = 5)	96.7	1.2	-
		Overall (n= 10)	93.1	4.7	-
Bovine urine	Zoxamide 336 > 159.0	0.01 (n = 5)	89.4	5.1	-
		0.1 (n = 5)	96.0	1.5	-
		Overall (n= 10)	92.7	5.1	-

Table A 142: Characteristics for the method used for validation of Zoxamide residues in body fluids and tissues

	Zoxamide
Specificity	Mass spectrum is provided. blank value < 30 % LOQ
Calibration (type, number of data points)	individual calibration data presented| calibration line equation presented number of data points: 5
Calibration range	Accepted calibration range in concentration units: 0.5 – 50 µg/L Corresponding calibration range in mass ratio units for the sample: 0.002 – 0.2 mg/kg for tissues and 0.002 – 0.2 mg/L for fluids
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	0.01 mg/kg for tissues 0.01 mg/L for fluids

Conclusion

The method is valid and acceptable according to SANTE/2020/12830 rev. 2 for the determination of Zoxamide in body fluids and tissues.

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

No new or additional studies have been submitted

A 2.2 Analytical methods for Potassium phosphonates

A 2.2.1 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.2.1.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

A 2.2.1.1.1 Analytical method 1

A 2.2.1.1.1.1 Method validation

No new or additional studies have been submitted

A 2.2.1.1.2 Analytical method 2

No new or additional studies have been submitted

A 2.2.1.1.2.1 Independent laboratory validation

No new or additional studies have been submitted

A 2.2.1.1.2.2 Confirmatory method (if required)

No new or additional studies have been submitted

A 2.2.1.1.2.3 Extraction efficiency

No new or additional studies have been submitted

A 2.2.1.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

Not relevant for this submission. No new or additional studies have been submitted

A 2.2.1.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted

A 2.2.1.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted

A 2.2.1.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted

A 2.2.1.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted

A 2.2.1.7 Other Studies/ Information

No new or additional studies have been submitted