

REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: FGF11

Product name(s): Telaron Alpha / Patton Alpha

Azoxystrobin 160 g/L + Prothioconazole 200 g/L SC

Chemical active substance(s):

Azoxystrobin, 160 g/L

Prothioconazole, 200 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(new authorization)

Applicant: UPL Holdings Coöperatief U.A.

Submission date: November 2024

MS Finalisation date: May 2025 (initial Core Assessment)

August 2025 (final Core Assessment)

Version history

When	What
November 2024	dRR Part B Section 5 - Submitted by the applicant
May 2025	Initial zRMS assessment The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are ~~struck through~~ and shaded for transparency. Following the evaluation and before sending the document for commenting, all coloured highlighting was removed, from the parts updated by the Applicant, for better legibility.
August 2025	Final report (Core Assessment updated following the commenting period). No additional information or assessments after the commenting period.

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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.

Noticed data gaps are: none.

Commodity/crop	Supported/Not supported
high starch content /cereals	Supported
high starch-oil content /oilseed rape	Supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of zRMS:	Study acceptable. The analytical method for the determination of azoxystrobin and prothioconazole in plant protection product was fully validated according to SANCO/3030/99 rev. 5.
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Reference: KCP 5.1.1-01

Report Validation of analytical method for determination of active ingredients of Azoxystrobin 160 + Prothioconazole 200 g/L SC; Patil, B., 2024; Study No. UPL/2023/1623

Guideline(s): Yes – SANCO/3030/99 revision 5

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The active ingredients content of Azoxystrobin 160 + Prothioconazole 200 g/L SC is determined by high performance liquid chromatography on a reversed phase column C-18 using Acetonitrile (40%): 0.1% Orthophosphoric acid (OPA) in Milli Q Water (60%), v/v, as mobile phase and UV detection at 250 nm.

Test item

Test Item Name	Azoxystrobin 160 + Prothioconazole 200 g/L SC
Sponsor's Test Item Code	FGF11/GPF 1021
IUPAC Name	Azoxystrobin: methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4- yloxy]phenyl}-3-methoxyacrylate Prothioconazole: 2-[(2RS)-2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)- 2-hydroxypropyl]-2H-1,2,4-triazole-3(4H)-thione

CA Name	Azoxystrobin: methyl (E)-2-[[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy]- α -(methoxymethylene)benzeneacetate Prothioconazole: 2-[2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-3H-1,2,4-triazole-3-thione
CAS Number	Azoxystrobin: [131860–33–8] Prothioconazole: [178928–70–6]
Molecular Weight	Azoxystrobin: 403.4 Prothioconazole: 344.3
Batch/Lot Number	ARD/AP/360/SC/36
Analysed Concentration	Azoxystrobin: $14.45 \pm 0.04\%$ w/w or 162.20 ± 0.48 g/L Prothioconazole: $17.86 \pm 0.06\%$ w/w or 200.54 ± 0.65 g/L
Date of Manufacture	May 24, 2023
Date of Expiry	May 23, 2025
Appearance	Light brown colour liquid suspension, free from visible extraneous matter

Reference standards

Reference Standard	Azoxystrobin
CAS Number	[131860-33-8]
Batch/Lot Number	BCCF6593
Molecular Formula	C ₂₂ H ₁₇ N ₃ O ₅
Date of Quality Release	March 25, 2021
Date of Expiry	February 2026
Purity	99.3% (Refer to Certificate of Analysis in APPENDIX 4)
Source	Sigma Aldrich
Storage Condition	Refrigerator
JRF Entry Number	PC-6041
Reference Standard	Prothioconazole
CAS Number	[178928-70-6]
Batch/Lot Number	G1292095
Molecular Formula	C ₁₄ H ₁₅ Cl ₂ N ₃ OS
Date of Certification	September 22, 2022
Date of Expiry	September 22, 2026
Purity	98.29% (Refer to Certificate of Analysis in APPENDIX 4)
Source	Dr. Ehrenstorfer
Storage Condition	Refrigerator
JRF Entry Number	PC-5964

Blank formulation

Reference Standard	Azoxystrobin
CAS Number	[131860-33-8]
Batch/Lot Number	BCCF6593
Molecular Formula	C ₂₂ H ₁₇ N ₃ O ₅
Date of Quality Release	March 25, 2021
Date of Expiry	February 2026
Purity	99.3% (Refer to Certificate of Analysis in APPENDIX 4)
Source	Sigma Aldrich
Storage Condition	Refrigerator
JRF Entry Number	PC-6041
Reference Standard	Prothioconazole
CAS Number	[178928-70-6]
Batch/Lot Number	G1292095
Molecular Formula	C ₁₄ H ₁₅ Cl ₂ N ₃ OS
Date of Certification	September 22, 2022
Date of Expiry	September 22, 2026
Purity	98.29% (Refer to Certificate of Analysis in APPENDIX 4)
Source	Dr. Ehrenstorfer
Storage Condition	Refrigerator
JRF Entry Number	PC-5964

Equipment/Instruments

S. N°	Name	Model	Make / Supplier
1	Balances	SECURA26P-10IN	Sartorius Lab instruments GmbH & Co.
		SECURA225D-10IN	Sartorius Lab instruments GmbH & Co.
2	HPLC	LC2030C	Shimadzu
3	Refrigerator	IL-725	Scineers Science + Engineers

Solvent/Chemicals

S. N°	Name	Grade	Source
1	Water	Milli-Q	Merck Milli Pore Milli-Q Water Purification System
2	Acetonitrile	HPLC	Merck
3	Orthophosphoric acid (OPA)	HPLC	Qualigens

Instrumental Parameters

Instrument	: HPLC (Shimadzu, LC2030C Prominence i with Lab solution software, Version 5.98SP 1)
Column	: Intersil ODS-2 C18 [150 mm x 4.6 mm (i.d.); 5.0 µm particle size]
Wavelength	: 250 nm
Mobile Phase	: 0.1% Orthophosphoric acid in Milli Q Water (60%): Acetonitrile (40%), v/v
Injection Volume	: 10 µL
Flow Rate	: 1.5 mL/minute
Column Temperature	: 40 °C
Diluent	: 0.1% Orthophosphoric acid (OPA) in Acetonitrile

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances in plant protection product FGF11

	Azoxystrobin	Prothioconazole
Author(s), year	Patil, 2024	Patil, 2024
Principle of method	HPLC-UV (250 nm)	HPLC-UV (250 nm)
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	51.20 to 190.58 mg/L (6.90 to 25.69 %w/w) Nominal concentration is 14.45 % w/w, since the lowest concentration is 6.90 % w/w and the highest concentration is 25.69 % w/w, the calibration range extends an appropriate range of $\pm$ at least 20%	51.58 to 252.53 mg/L (6.95 to 34.04 %w/w) Nominal concentration is 17.86 % w/w, since the lowest concentration is 6.95 % w/w and the highest concentration is 34.04 % w/w, the calibration range extends an appropriate range of $\pm$ at least 20%
Precision – Repeatability Mean n = 5 (%RSD)	%RSD = 0.21 Extended Horwitz = 1.79 Horrat Value (Hr) = 0.12	%RSD = 0.21 Extended Horwitz = 1.79 Horrat Value (Hr) = 0.12
Accuracy n = 5 (% Recovery)	total recovery 100.21 % (at 82.78 mg/L) 101.34 % (at 104.27 mg/L) 100.10% (at 123.97 mg/L)	total recovery 100.10 % (at 102.34 mg/L) 101.68 % (at 130.06 mg/L) 100.82% (at 155.77 mg/L)
Interference/ Specificity	No significant interferences were noted from the chromatograms (comparison of blank and fortified samples).	
Comment	The method is fit for its intended purpose.	

Conclusion

The analytical method provides a specific determination of the active ingredients prothioconazole and azoxystrobin in the formulation FGF11 and fulfils the requirements of SANCO/3030/99 rev.5.

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

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

Comments of zRMS:	Study acceptable The analytical method for the determination of relevant impurities in plant protection product was fully validated according to SANCO/3030/99 rev. 5.
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Reference: KCP 5.1.1-02

Report Validation of analytical method for determination of associated impurity (z-isomer of azoxystrobin) of Azoxystrobin 160 + Prothioconazole 200 g/L SC; Patil, B., 2024; Study No. UPL/2023/1628

Guideline(s): Yes – SANCO/3030/99 revision 5

Deviations: No

GLP: Yes

Acceptability: Yes

Reference: KCP 5.1.1-03

Report Validation of analytical method for determination of associated impurity (toluene) of Azoxystrobin 160 + Prothioconazole 200 g/L SC; Patil, B., 2024; Study No. UPL/2023/1629

Guideline(s): Yes – SANCO/3030/99 revision 5

Deviations: No

GLP: Yes

Acceptability: Yes

Reference: KCP 5.1.1-04

Report Validation of analytical method for determination of associated impurity (prothioconazole-desthio) of Azoxystrobin 160 + Prothioconazole 200 g/L SC; Patil, B., 2024; Study No. UPL/2023/1630

Guideline(s): Yes – SANCO/3030/99 revision 5

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The impurity content (Z-isomer of Azoxystrobin) of FGF11 is determined by high performance liquid chromatography on a reversed phase column (C-18) using Acetonitrile (50%) : 0.01% v/v Orthophosphoric acid (OPA) in Milli-Q Water (50 %)(v/v) as mobile phase and UV detection at 220 nm.

The impurity content (Toluene) of FGF11 is determined by high performance liquid chromatography on a reversed phase column (C-18) using Solvent A- 0.05% v/v Orthophosphoric acid (OPA) in Milli-Q Water: Solvent B- Methanol (Gradient) as mobile phase and UV detection at 210 nm.

The impurity content (Prothioconazole-desthio) in FGF11 is determined by high performance liquid chromatography on a reversed phase column (C-18) using Solvent A- 0.05% v/v Orthophosphoric acid (OPA) in Milli-Q Water: Solvent B- Methanol (Gradient) as mobile phase and UV detection at 210 nm.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) FGF11

	Z-isomer*	Toluene*	Prothioconazole-desthio*
Author(s), year	Patil, 2024	Patil, 2024	Patil, 2024
Principle of method	HPLC-UV (220 nm)	HPLC-UV (210 nm)	HPLC-UV (210 nm)
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	0.02 to 33.77 mg/L 0.00002 to 0.03362 %w/w	0.04 to 45.41 mg/L 0.00004 to 0.04522 %w/w	0.06 to 5.96 mg/L 0.000060 to 0.005937 %w/w
Precision – Repeatability Mean n = 5 (%RSD)	%RSD = 1.88 Extended Horwitz = 6.22 Horrat Value (H _r) = 0.3	%RSD = 0.34 Extended Horwitz = 4.72 Horrat Value (H _r) = 0.07	%RSD = 0.34 Extended Horwitz = 6.27 Horrat Value (H _r) = 0.36
Accuracy n = 5 (% Recovery)	100.21 % (at 0.03 mg/L) 100.27 % (at 0.3 mg/L)	101.14 % (at 0.08 mg/L) 98.35 % (at 0.8 mg/L)	95.63 % (at 0.14 mg/L) 93.47 % (at 1.4 mg/L)
Interference/ Specificity	No significant interferences were noted from the chromatograms (comparison of blank and fortified samples).		
LOQ	0.03 mg/L	0.08 mg/L	0.14 mg/L

	Z-isomer*	Toluene*	Prothioconazole-desthio*
Comment	The methods are fit for their intended purpose.		

* Limits are provided in Part B1, Section 1.4.1.

Conclusion

The analytical method provides a specific determination of the relevant impurities in the formulation FGF11 and fulfils the requirements of SANCO/3030/99 rev.5.

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

None of the formulants is of toxicological, environmental or ecotoxicological relevance within the formulation FGF11. Therefore, no analytical method is required.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For technical Azoxystrobin a CIPAC method is available: CIPAC method 571
For Prothioconazole no CIPAC methods are available.

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 azoxystrobin and prothioconazole for the generation of pre-authorization data is given in the following table. For the detailed evaluation of new studies it is referred to Appendix 2.

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

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products – Wheat grain and straw (Azoxystrobin)	Primary	0.01 mg/kg	LC-MS/MS	S. Richter & T. Stanislawski, 2021, Report No. VV-892169 ***
Plants, plant products – Barley grain and straw (Azoxystrobin)	Primary	0.01 mg/kg	LC-MS/MS	S. Richter & T. Stanislawski, 2021, Report No. VV-892169 ***
Honey (TDMs)	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.1.2/13 Frédéric Lebrun, 2024, Report No. 644-2023
Plants, plant products – Oilseed rape seed, whole plant (Azoxystrobin, z-Azoxystrobin)	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.1.2/04 T. Rastogi, 2024, Report No. UPL/2023/1991
Plants, plant products – Wheat grain and straw (Prothioconazole - desthio*)	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.1.2/01 J. Pearson, 2022, Report No. QG/20/011
Plants, plant products – Oilseed rape seed, field bean (dried), barley grain, barley forage, cucumber, orange, oilseed rape forage, wheat forage, wheat grain, barley straw,	Primary	0.005 mg/kg	LC-MS/MS	KCP 5.1.2/02 D. Giancola, 2023, Report No. QG/22/001

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
wheat straw (Prothioconazole- α -hydroxy-desthio, Prothioconazole-3-hydroxy-desthio, Prothioconazole-4-hydroxy-desthio, Prothioconazole-5-hydroxy-desthio, Prothioconazole-6-hydroxy-desthio)				
Plants, plant products – Barley grain, barley, plant, barley straw, cucumber, field bean, oilseed rapeseed, orange, wheat grain, wheat plant, wheat straw (TDMs**)	Primary	0.005-0.2 mg/kg	LC-MS/MS	KCP 5.1.2/03 B. Phipps, 2024, Report No. QG/20/012
Ecotoxicology – Water media (Azoxystrobin)	Primary	0.006 mg test item/L	LC-MS/MS	KCP 5.1.2/09 J. Bauer & G. Mollandin, 2024; Study No.: UPL/2024/0581
	Primary	0.01 mg test item/L	LC-MS/MS	KCP 5.1.2/10 J. Bauer & G. Mollandin, 2024; Study No.: UPL/2024/0702
	Primary	13 g test item/L	HPLC-UV	KCP 5.1.2/11 S. Stürtz & C. Horstmann, 2024; Study No.: UPL/2023/2350
	Primary	13 g test item/L	HPLC-UV	KCP 5.1.2/12 S. Stürtz & C. Horstmann, 2024; Study No.: UPL/2023/2349
Ecotoxicology – Water media (Prothioconazole)	Primary	0.006 mg test item/L	LC-MS/MS	KCP 5.1.2/09 J. Bauer & G. Mollandin, 2024; Study No.: UPL/2024/0581
	Primary	0.01 mg test item/L	LC-MS/MS	KCP 5.1.2/10 J. Bauer & G. Mollandin, 2024; Study No.: UPL/2024/0702
	Primary	13 g test item/L	HPLC-UV	KCP 5.1.2/11 S. Stürtz & C. Horstmann, 2024; Study No.: UPL/2023/2350
	Primary	13 g test item/L	HPLC-UV	KCP 5.1.2/12 S. Stürtz & C. Horstmann, 2024; Study No.: UPL/2023/2349
Ecotoxicology – Bee feeding / contact solutions (Azoxystrobin)	Primary	2.5 mg/L	HPLC-UV	KCP 5.1.2/05 C. Horstmann, 2024; Study No.: UPL/2023/1657
	Primary	7.3 mg Azoxystrobin/L (contact) 8.7 mg Azoxystrobin/L (oral)	HPLC-UV	KCP 5.1.2/06 C. Horstmann, 2024; Study No.: UPL/2023/1780
	Primary	9.2 mg /L (contact) 7.2 mg /L (oral)	HPLC-UV	KCP 5.1.2/07 C. Horstmann, 2024; Study No.: UPL/2023/2345

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Primary	18 mg/L	HPLC-UV	KCP 5.1.2/08 S. Venturi, 2024; Study No.: UPL/2023/2016
Ecotoxicology – Bee feeding / contact solutions (Prothioconazole)	Primary	3.1 mg/L	HPLC-UV	KCP 5.1.2/05 C. Horstmann, 2024; Study No.: UPL/2023/1657
	Primary	9.0 mg /L (contact) 10.8 mg /L (oral)	HPLC-UV	KCP 5.1.2/06 C. Horstmann, 2024; Study No.: UPL/2023/1780
	Primary	11.4 mg/L (contact) 8.9 mg /L (oral)	HPLC-UV	KCP 5.1.2/07 C. Horstmann, 2024; Study No.: UPL/2023/2345
	Primary	20 mg/L	HPLC-UV	KCP 5.1.2/08 S. Venturi, 2024; Study No.: UPL/2023/2016

* Sum of prothioconazole-desthio and all metabolites containing the 2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl-2H-1,2,4-triazole moiety, expressed as prothioconazole-desthio (sum of isomers).
 ** Triazole alanine (TA), triazole lactic acid (TLA), Triazole acetic acid (TAA), and 1,2,4-triazole (1,2,4-T).
 *** The study VV-892169 has been referenced in this dossier but has been submitted for the purposes of the azoxystrobin renewal (please refer to the assessment and conclusions of the EU renewal for azoxystrobin). UPL has access to rely on the data contained within this study report to support the registration of FGF11.
 Test item = formulated product (FGF11) – relevant to the ecotoxicology studies referenced in the above table.

zRMS comments:

The Applicant submitted analytical methods for the determination of prothioconazole and azoxystrobin residues in different matrices for the generation of pre-authorization data. The methods are acceptable. The details of the evaluation of new and additional studies are referred in Appendix 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 Azoxystrobin (KCP 5.2)

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

The residue definitions given in the following table are identical to those proposed in the EFSA Peer review of the pesticide risk assessment -EFSA Journal 2010;8(4):1542.

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	Azoxystrobin	LOQ = 0.01 mg/kg / MRL=0.01* mg/kg Relevant only for cereal (wheat, barley, and rye) and OSR forage – EU MRLs not set for livestock feed items.	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg Not relevant for the intended uses of this product	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Plant, high protein/high starch content (dry commodities)		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg MRLs for cereal grain: 0.5 mg/kg (wheat grain) 1.5 mg/kg (barley grain) 0.5 mg/kg (rye grain) Also covers straw, but EU MRLs are not currently set for livestock feed items.	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Plant, high oil content		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg MRL = 0.7 mg/kg (OSR seed)	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Muscle	Azoxystrobin	LOQ = 0.01 mg/kg / MRL=0.01* mg/kg	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Milk		LOQ = 0.001 mg/kg / MRL=0.01* mg/kg	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Eggs		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Fat		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Liver, kidney		LOQ = 0.01 mg/kg / MRL=0.01* mg/kg	EFSA Journal 2010;8(4):1542 / Reg. (EU) 2024/2633
Soil (Ecotoxicology)	Azoxystrobin	LOQ = 0.02 mg/kg	EFSA Journal 2010;8(4):1542
Drinking water (Human toxicology)	Azoxystrobin	LOQ = 0.1 µg/L	EFSA Journal 2010;8(4):1542
Surface water (Ecotoxicology)	Azoxystrobin	LOQ = 0.1 µg/L	EFSA Journal 2010;8(4):1542
Air	Azoxystrobin	LOQ = 3 µg/m ³	EFSA Journal 2010;8(4):1542
Tissue (meat or liver)	Azoxystrobin	LOQ = 0.01 mg/kg	EFSA Journal 2010;8(4):1542 / SANTE/2020/12830, Rev.2
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev.12

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

Analytical methods for the determination of residues in plant matrices were already evaluated during the EU Review of Azoxystrobin (EFSA Journal 2010;8(4):1542). An overview on the acceptable methods and possible data gaps for analysis of Azoxystrobin in plant matrices is given in the following table.

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: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
High acid content	Primary	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
High oil content	Primary	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
High protein/high starch content (dry)	Primary	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	LC-MS/MS	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Letter of Access submitted separately with this application
Not required, because:	- According to SANTE 2017/10632 Rev. 4, 23 February 2022, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required. Azoxystrobin is currently under a second renewal process which is not finalised yet – consequently, this should not be required.

zRMS comments:

The methods available for azoxystrobin in plant matrices were sums in EFSA document “Review of the existing MRLs for azoxystrobin” (EFSA Journal 2013;11(12):3497):

“1. Methods for enforcement of residues in food of plant origin

During the renewal peer review under Directive 91/414/EC, the multi-residue method DFG S 19 using HPLC-MS/MS and its ILV were evaluated and validated in plant matrices for the determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in dry (cereals grain), acidic (orange), high water content (lettuce) and high oil content (oilseed rape) commodities (United Kingdom, 2009a, 2009b; FAO, 2008).

Furthermore, an analytical method using HPLC-MS/MS and its ILV were evaluated and adequately validated in plant matrices for the determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in dry (wheat, barley grain), acidic (grape, mandarin, orange), high water content (tomato, lettuce, cabbage, carrot, kale, potato) and high oil content (avocado, sunflower seed, oilseed rape,) commodities, and in hops (United Kingdom, 2009b; FAO, 2008).

The multi-residue QuEChERS methods in combination with HPLC-MS/MS and GC/MS, as described by CEN (2008), are also available to analyse parent azoxystrobin but validation data were not evaluated in detail because a validated analytical method is reported above.

Hence, it is concluded that parent azoxystrobin can be enforced in food of plant origin with an LOQ of 0.01 mg/kg in dry, acidic, high water content and high oil content commodities, and in hops. As the active substance does not contain a chiral center, the analytical method is considered as specific to the active substance. Therefore, no further consideration of monitoring methods for plant matrices is necessary.

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

Analytical methods for the determination of residues in animal matrices were already evaluated during the EU Review of Azoxystrobin (EFSA Journal 2010;8(4):1542). An overview on the acceptable methods and possible data gaps for analysis of Azoxystrobin in animal matrices is given in the following table.

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

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.001 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	ILV	0.001 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
Eggs	Primary	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
Muscle	Primary	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
Fat	Primary	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required
Kidney, liver	Primary	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	ILV	0.01 mg/kg	GC-NPD	EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	-	-	Not required

Honey

Letter of Access submitted separately with this application

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	-
Not required, because:	Residues above LOQ are not expected.

zRMS comments:

The methods available for azoxystrobin in animal matrices were sums in EFSA document “Review of the existing MRLs for azoxystrobin” (EFSA Journal 2013;11(12):3497):
“2. Methods for enforcement of residues in food of animal origin
During the peer review under Directive 91/414/EEC, an analytical method using GC-NPD and its ILV were

evaluated and validated for determination of parent azoxystrobin with an LOQ of 0.001 mg/kg in milk and 0.01 mg/kg in eggs, liver, fat, muscle. Nevertheless, no confirmatory method was available (United Kingdom, 2009a; FAO, 2008).

Furthermore, an analytical method using HPLC-MS/MS and its ILV were evaluated in the JMPR report and validated in food of animal origin for determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in muscle, fat, milk, kidney, liver and eggs (FAO, 2008).

Hence, it is concluded, that parent azoxystrobin can be enforced in food of animal origin with an LOQ of at least 0.01 mg/kg in muscle, fat, milk, kidney, liver and eggs.”

Therefore, no further consideration of monitoring methods for animal matrices is necessary.

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

Analytical method for the determination of residues in soil was already evaluated during the EU Review of Azoxystrobin (EFSA Journal 2010;8(4):1542). An overview on the acceptable methods and possible data gaps for analysis of Azoxystrobin in soil is given in the following table.

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

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.02 mg/kg	HPLC-MS/MS HPLC-UV GC-MSD	EFSA Journal 2010;8(4):1542
Confirmatory	-	-	Not required

zRMS comments:

Adequate methods are available for the determination of residues of azoxystrobin in soil. Therefore, no further consideration of monitoring methods for soil is necessary.

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

Analytical methods for the determination of residues in water matrices were already evaluated during the EU Review Azoxystrobin (EFSA Journal 2010;8(4):1542). An overview on the acceptable methods and possible data gaps for analysis of Azoxystrobin in surface and drinking water is given in the following table.

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

Component of residue definition: Azoxystrobin				
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	GC-MSD	EFSA Journal 2010;8(4):1542
	Confirmatory	-	-	Not required
	ILV	-	-	Required
Surface water	Primary	0.1 µg/L	GC-MSD	EFSA Journal 2010;8(4):1542
	Confirmatory	-	-	Not required

An independent laboratory validation of the drinking water method was not evaluated during the EU Review of Prothioconazole (EFSA Scientific Report (2007) 106, 1-98). This information will be submitted during the renewal of the active substance.

UPL has data access to the Annex II (active substance) studies submitted by Syngenta for the purposes of the renewal, which includes the following studies to address this data point. If necessary, reference is made to these studies which are currently being evaluated as part of the ongoing active substance renewal for azoxystrobin:

- Method to quantify azoxystrobin in water, using LC-MS/MS, LOQ = 0.05 µg/L, Amic 2012, S11-03538, VV-401211
- Method (ILV) to quantify azoxystrobin in water, using LC-MS/MS, LOQ = 0.05 µg/L, Brown 2019, RES-00193, VV-619234

zRMS comments:

In “Peer Review of the pesticide risk assessment of the active substance azoxystrobin” (EFSA Journal 2010; 8(4):1542) it is stated that “*Monitoring of residues of azoxystrobin in groundwater, drinking water and surface water can be done by GC-MSD. Pending on the data gap identified in section 4, the residue definition for water might change and therefore further methods could be required in the future.*”

ILV for the analytical method for drinking water is required (data gap).

Applicant has data access to analytical method (Amic 2012, S11-03538, VV-401211) and its ILV (Brown 2019, RES-00193, VV-619234) to quantify azoxystrobin in water using LC-MS/MS with LOQ = 0.05 µg/L. These methods are currently being evaluated as part of the ongoing active substance renewal for azoxystrobin.

Table 5.3-8: Validated methods for water

Component of residue definition: azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking and surface water	Primary (Method GRM057.01A)	0.05 µg/L ^{a)}	LC-MS/MS (2 transitions)	<i>Method:</i> Amic S., 2012 / Report No. GRM057.01A / (VV-128281) <i>Validation:</i> Amic S., 2012a / Report No. S11-03538 / (VV-401211)
Drinking water	ILV (Method GRM057.01A)	0.05 µg/L ^{a)}	LC-MS/MS (2 transitions)	Brown D., 2019 / Report No. RES-00193 / (VV-619234)

a) Method also validated for metabolite R234886 (LOQ 0.05 µg/L)

These studies have not been reassessed in the framework of this application.

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

Analytical method for the determination of residues in air was already evaluated during the EU Review of Azoxystrobin (EFSA Journal 2010;8(4):1542). An overview on the acceptable methods and possible data gaps for analysis of Azoxystrobin in air is given in the following table.

Table 5.3-9: Validated methods for air

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	3 µg/m ³	GC-MSD	EFSA Journal 2010;8(4):1542
Confirmatory	-	-	Not required

zRMS comments:

Adequate method is available for the determination of residues of azoxystrobin in air. Therefore, no further consideration of monitoring methods for air is necessary.

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

Letter of Access submitted separately with this application.

zRMS comments:

Adequate method is available for the determination of residues of azoxystrobin in human whole blood. Applicant

has data access to analytical method of Gemrot F., 2011 / Report S10-03815 (VV-398250).

Table 5.3-10: Methods for body fluids and tissues

Component of residue definition: azoxystrobin				
Matrix	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / Report No. / EU agreed
Human whole blood	Primary (Method RAM 399/01)	0.01 µg/mL	LC-MS/MS	Method and validation: Gemrot F., 2011 / Report S10-03815 (VV-398250)

Therefore, no further consideration of monitoring method for body fluids is necessary.

5.3.2.8 Other studies/ information

No further studies are required.

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

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

Table 5.3-11: 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 – cereal and oilseed rape (OSR) forage	Prothioconazole-desthio (sum of isomers)	0.01* mg/kg <i>Relevant only for cereal (wheat, barley, and rye) and OSR forage – EU MRLs not set for livestock feed items.</i>	Reg. (EU) 2024/1318
Plant, high acid content		0.01 mg/kg* <i>Not relevant for the intended uses of this product</i>	Reg. (EU) 2024/1318
Plant, high protein/high starch content (dry commodities) – cereal (grain)		0.01* mg/kg 0.1 mg/kg (wheat grain) 0.2 mg/kg (barley grain) 0.05 mg/kg (rye grain) Also covers straw, but EU MRLs are not currently set for livestock feed items.	Reg. (EU) 2024/1318
Plant, high oil content		0.02 mg/kg* 0.15 mg/kg (OSR seed)	Reg. (EU) 2024/1318
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg* <i>Not relevant for the intended uses of this product</i>	Reg. (EU) 2024/1318
Muscle	Prothioconazole-desthio (sum of isomers)	0.01 mg/kg	Reg. (EU) 2024/1318
Milk		0.01* mg/kg	Reg. (EU) 2024/1318
Eggs		0.01* mg/kg	Reg. (EU) 2024/1318
Fat		0.01* mg/kg	Reg. (EU) 2024/1318
Liver, kidney		0.1 mg/kg	Reg. (EU) 2024/1318
Honey		0.05* mg/kg	Reg. (EU) 2024/1318
Soil	Prothioconazole and	0.05 mg/kg	common limit

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
(Ecotoxicology)	prothioconazole-desthio		
Drinking water (Human toxicology)	Prothioconazole and prothioconazole-desthio	0.1 µg/L	General EU limit for drinking water
Surface water (Ecotoxicology)	Prothioconazole and prothioconazole-desthio	RAC 13 µg/L (prothioconazole) RAC 0.334 µg/L (prothioconazole-desthio)	from EC50 for <i>Daphnia magna</i> (ESFA Scientific Report, 2007) from NOEC for <i>Oncorhynchus mykiss</i> (ELS) (ESFA Scientific Report, 2007)
Air	Prothioconazole and prothioconazole-desthio	LOQ = 0.15 µg/m ³ (prothioconazole) LOQ = 0.0006 µg/m ³ (prothioconazole- desthio)	EFSA Scientific Report (2007)
Tissue (meat or liver)	prothioconazole-desthio	0.01 mg/kg	SANTE/2020/12830, Rev. 2
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev. 2

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

Table 5.3-12: 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: prothioconazole-desthio				
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	0.05 mg/kg (wheat, forage) 0.05 mg/kg (barley, forage) 0.02 mg/kg (tomato)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (cucumber, wheat forage, barley forage)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011
	ILV	0.02 mg/kg (tomato fruit)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (cucumber)	LC-MS/MS	N. Boubakri, 2023, Report No. S21- 08354
	Confirmatory	Not required		
High acid content	Primary	0.02 mg/kg (orange)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (orange)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011
	ILV	0.02 mg/kg	GC-MS	DAR prothioconazole, 2005, EU

		(tomato fruit)		agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (orange)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354
	Confirmatory	Not required		
High oil content	Primary	0.02 mg/kg (rapeseed)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011
	ILV	0.02 mg/kg (rape seed)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354
	Confirmatory	Not required		
High protein/high starch content (dry)	Primary	0.05 mg/kg (wheat, straw) 0.02 mg/kg (wheat, grain) 0.05 mg/kg (barley, straw) 0.02 mg/kg (barley, grain)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (dried field bean, wheat straw, barley straw)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011
	ILV	0.02 mg/kg (cereal grain)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (dried field bean)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354
	Confirmatory	Not required		
Difficult (if required, depends on intended use)	Primary	Not relevant for the intended uses of this product		

Table 5.3-13: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	DAR – Prothioconazole 2005. Extraction efficiency was demonstrated. Extraction efficiency of the residue method in cereals and rape (Heinemann, O) was evaluated using aged radioactive residues from the metabolism study on wheat (Haas, M). In summary, the comparison of the residue analytical method of extraction for plant matrices with the extraction method used in the metabo-lism study demonstrated the suitability of the analytical method (extracting with an acetonitrile/water solvent system) for the determination of the relevant residue in plant matrices. No further consideration was determined to be necessary and the extraction will be reevaluated at renewal.

zRMS comments:

The EU agreed methods by Weeren and Pelz (2000) and Class (2001) are not sufficient to monitor lowered MRLs than 0.02 mg/kg for food of plant origin.
To cover the current residue definition and MRL limits, the applicant provided sufficiently validated analytical

method of J. Pearson (2022, Report No. QG/20/011) and the ILV of QG/20/011 (N. Boubakri, 2023, Report No. S21-08354) for the determination of relevant residues of prothioconazole-desthio in/on matrices of plant origin (high water content, high acid, high oil content and high protein/high starch content) with LOQ of 0.005 mg/kg. The analytical method of Pearson (2022, Report No. QG/20/011) was successfully independently validated. The methods are acceptable.
The details of the evaluation of new and additional studies are referred in Appendix 2.

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

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

Table 5.3-14: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: prothioconazole-desthio*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.004 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.004 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Eggs	Primary	0.01 mg/kg	LC-MS/MS	D. Kleinhenz, 2023, Report No. S21-08355
	ILV	0.01 mg/kg	LC-MS/MS	T. Rastogi, 2023, Report No. S21-08868
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Fat	Primary	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Honey	Primary	0.005 mg/kg	LC-MS/MS	J. Hitchens, 2023, Report No. QG/21/009
	ILV	0.005 mg/kg	LC-MS/MS	J. Wagner, 2023, Report No. S21-08357

*An alternative residue definition for animal matrices was proposed the EFSA Scientific Report 2007: Sum of prothioconazole-desthio and its glucuronic conjugate, expressed as prothioconazole-desthio. However, in the EFSA Art 12 MRL review 2014, the residue definition was instead proposed as prothioconazole-desthio.

For any special comments or remarkable points concerning the analytical methods for the determination

of residues in animal matrices, please refer to Appendix 2.

Table 5.3-15: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	DAR – Prothioconazole 2005. Extraction efficiency was demonstrated. Extraction efficiency of the residue method in animal matrices (Heinemann, O) was evaluated using aged radioactive residues from the metabolism study in goats (Weber, H., Weber, E., and Spiegel, K.). In summary, the comparison of the residue analytical method of extraction for animal matrices with the extraction method used in the metabolism study demonstrated the suitability of the analytical method (extracting with an acetonitrile/water solvent system) for the determination of the relevant residue in animal matrices. No further consideration was determined to be necessary and the extraction will be reevaluated at renewal.

zRMS comments:

During the peer review under Directive 91/414/EEC, an analytical method using HPLC-MS/MS and its ILV were evaluated and validated for the determination of prothioconazole-desthio only in food of animal origin with an LOQ of 0.004 mg/kg in milk and an LOQ of 0.01 mg/kg in muscle, fat, liver and kidney (United Kingdom, 2004; EFSA, 2007b). Hence it is concluded that prothioconazole-desthio can be enforced in food of animal origin with an LOQ of 0.004 mg/kg in milk and an LOQ of 0.01 mg/kg in muscle, fat, liver and kidney. Nevertheless, prothioconazole-desthio cannot be enforced in eggs. Therefore, a fully validated analytical method for the determination of prothioconazole-desthio in eggs is required.

According to the conclusions presented in EFSA Journal 2014;12(5):3689, a fully validated analytical method for the determination of prothioconazole-desthio in eggs is required.

The Applicant provided new method (D. Kleinhenz, 2023, Report No. S21-08355) validated for the determination of prothioconazole-desthio (as sum of isomers) in five (milk, egg, fat, liver and meat) different matrices of food of animal origin with the LOQ of 0.004 mg/kg for milk and 0.01 mg/kg for remaining matrices and its ILV (T. Rastogi, 2023, Report No. S21-08868) for the determination of prothioconazole-desthio residues in milk and fat with LOQ of 0.004 mg/kg and 0.01 mg/kg, respectively.

Additionally, analytical method and its ILV for determination of prothioconazole-desthio in honey is available. No additional data are required.

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

Analytical methods for the determination of residues in soil were already evaluated during the EU review (EFSA Scientific Report (2007) 106, 1-98). An overview on the acceptable methods are detailed below:

Table 5.3-16: Validated methods for soil

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.006 mg/kg	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Primary	0.01 mg/kg (prothioconazole-desthio only)	GC-MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
Primary	0.006 mg/kg (prothioconazole) 0.002 mg/kg (prothioconazole-desthio)	LC-MS/MS	M. Kaiser, 2022, Report No. S21-08358

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

Appendix 2.

zRMS comments:

Analytical method for determination of prothioconazole-desthio in soil is available.
Additionally the Applicant provided analytical method for determination of prothioconazole with LOQ of 0.006 mg/kg and prothioconazole-desthio with 0.002 mg/kg in soil.
The details of the evaluation of additional studies are referred in Appendix 2.
No additional data are required.

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

Table 5.3-17: Validated methods for water

Component of residue definition: prothioconazole and prothioconazole-desthio				
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 (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.05 µg/L	LC-MS/MS	D. Kaiser, 2022, Report No. S21-08359
	ILV	0.05 µg/L	LC-MS/MS	S. Jooß, 2023, Report No. S21-08869
Surface water	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)
	Primary	0.05 µg/L	LC-MS/MS	D. Kaiser, 2022, Report No. S21-08359

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

zRMS comments:

Analytical method for determination of prothioconazole and prothioconazole-desthio in water is available (EFSA, 2007).
Additionally, an independent laboratory validation (ILV) for the method for the determination of residues of prothioconazole in drinking water is missing (EFSA, 2007). Based on the indication of the SANTE/2020/12830, Rev.2, 14. February 2023, the ILV for drinking water should be submitted.
Applicant provided the LC-MS/MS analytical method (D. Kaiser, 2022, Report No. S21-08359) with its ILV (S. Jooß, 2023, Report No. S21-08869) for the determination of prothioconazole and prothioconazole-desthio in drinking water with LOQ of 0.05 µg/L.
The details of the evaluation of additional studies are referred in Appendix 2.
No additional data are required.

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

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

Table 5.3-18: Validated methods for air

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.015 µg/L (prothioconazole) 0.0006 µg/L (prothioconazole-desthio)	HPLC-MS/MS	DAR prothioconazole, 2005, EU agreed (EFSA Scientific Report, 2007)

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

zRMS comments:

Analytical method for determination of prothioconazole and prothioconazole-desthio in air is available.
No additional data are required.

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole 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 5.3-19: Methods for body fluids and tissues (if appropriate)

Component of residue definition: prothioconazole-desthio			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/L	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08361

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

zRMS comments:

According to the EFSA Scientific Report (2007) 106, 1-98, Conclusion on the peer review of Prothioconazole, the point regarding analytical methods for body fluids and tissues for prothioconazole is open, data will be required if ECB classify the active substance as toxic. In Regulation (EU) No 283/2013 it is stated that “...methods, with a full description, shall be submitted for the analysis in body fluids and tissues for the active substance and relevant metabolites” and this is a new requirement of SANTE/2020/12830. According to the SANTE/2020/12830: “Analytical methods for monitoring residues in body fluids and tissues are required for detection of active substances and/or metabolites in humans and animals after possible intoxications or for biomonitoring purposes, regardless of their toxicological classification.”

Therefore, an analytical method for the residues of prothioconazole in body fluids and tissues is required.

The Applicant provided analytical method (N. Boubakri, 2023, Report No, S21-08361) validated for the determination of prothioconazole-desthio in urine with the LOQ of 0.01 mg/L.

The details of the evaluation of additional studies are referred in Appendix 2.

No additional data are required.

5.3.3.8 Other studies/ information

No further studies are 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	Vertebrate study Y/N	Owner
KCP 5.1.1-01	Patil, B.	2024	Title - Validation of analytical method for determination of active ingredients of Azoxystrobin 160 + Prothioconazole 200 g/L SC Company Report No UPL/2023/1623 GLP Unpublished	N	UPL
KCP 5.1.1-02	Patil, B.	2024	Title - Validation of analytical method for determination of associated impurity (z-isomer of azoxystrobin) of Azoxystrobin 160 + Prothioconazole 200 g/L SC Company Report No UPL/2023/1628 GLP Unpublished	N	UPL
KCP 5.1.1-03	Patil, B.	2024	Title - Validation of analytical method for determination of associated impurity (toluene) of Azoxystrobin 160 + Prothioconazole 200 g/L SC Company Report No UPL-2023-1629 GLP Unpublished	N	UPL
CP 5.1.1-04	Patil, B.	2024	Title - Validation of analytical method for determination of associated impurity (prothioconazole-desthio) of Azoxystrobin 160 + Prothioconazole 200 g/L SC Company Report No UPL/2023/1630 GLP Unpublished	N	UPL
KCP 5.1.2-01	Pearson, J.	2022	Title - Prothioconazole-desthio: Method Validation in Crops Company Report No QG/20/011 GLP Unpublished	N	UPL
KCP 5.1.2-02	Giancola, D.	2023	Title - Hydroxy Metabolites of prothioconazole-desthio: Method Validation in Crops Company Report No QG/22/001 GLP Unpublished	N	UPL
KCP 5.1.2-03	Phipps, B.	2024	Title - Triazole Derived Metabolites (TDM's) (1,2,4-triazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops	N	UPL

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Company Report No QG/20/012 GLP Unpublished		
KCP 5.1.2/04	Rastogi, T.	2024	Title - Validation of the Analytical Method for Determination of Azoxystrobin in OSR WholePlant and OSR Seeds Company Report No UPL/2023/1991 GLP Unpublished	N	UPL
KCP 5.1.2/05	Horstmann, C.	2024	Title - Azoxystrobin 160 + Prothioconazole 200 g/L SC: Chronic Oral Toxicity Test on the Honey Bee (Apis mellifera L.) in the Laboratory Company Report No UPL/2023/1657 GLP Unpublished	N	UPL
KCP 5.1.2/06	Horstmann, C.	2024	Title - Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects (Acute Contact and Oral) on Honey Bees (Apis mellifera L.) in the Laboratory Company Report No UPL/2023/1780 GLP Unpublished	N	UPL
KCP 5.1.2/07	Horstmann, C.	2024	Title - Azoxystrobin 160 g/L + Prothioconazole 200 g/L SC: Acute Contact and Oral Toxicity to Bumblebees (Bombus terrestris L.) in the Laboratory Company Report No UPL/2023/2345 GLP Unpublished	N	UPL
KCP 5.1.2/08	Venturi, S.	2024	Title - Effects of Azoxystrobin 160 + Prothioconazole 200 g/L SC on honeybees (Apis mellifera L.) 22-day larval toxicity test with repeated exposure Company Report No UPL/2023/2016 GLP Unpublished	N	UPL
KCP 5.1.2/09	Bauer, J. & Mollandin, G.	2024	Title - Azoxystrobin 160g/L + Prothioconazole 200g/L SC: Toxicity to Pseudokirchneriella subcapitata in an Algal Growth Inhibition Test Company Report No UPL/2024/0581 GLP Unpublished	N	UPL
KCP 5.1.2/10	Bauer, J. & Mollandin, G.	2024	Title - Azoxystrobin 160 g/L + Prothioconazole 200 g/L SC: Acute Toxicity to Daphnia magna in a Semi-Static 48-hour Immobilisation Test	N	UPL

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Company Report No UPL/2024/0702 GLP Unpublished		
KCP 5.1.2/11	Stürtz, S. & Horstmann, C.	2024	Title - Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects on Terrestrial (Non-Target) Plants: Vegetative Vigour Test Company Report No UPL/2023/2350 GLP Unpublished	N	UPL
KCP 5.1.2/12	Stürtz, S. & Horstmann, C.	2024	Title - Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects on Terrestrial (Non-Target) Plants: Seedling Emergence and Seedling Growth Test Company Report No UPL/2023/2349 GLP Unpublished	N	UPL
KCP 5.1.2/13	Frédéric Lebrun	2024	Magnitude of the residue of triazole derivative metabolites in phacelia honey in Northern and Southern Europe – 2023 Company Report No 644-2023 GLP Unpublished	N	UPL
KCP 5.2/01	Boubakri, N.	2023	Title - Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Prothioconazole-desthio in Different Matrices of Plant Origin Company Report No S21-08354 GLP Unpublished	N	UPL
KCP 5.2/02	Kleinhenz, D.	2023	Title - Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Food of Animal Origin Company Report No S21-08355 GLP Unpublished	N	UPL
KCP 5.2/03	Rastogi, T.	2023	Title - Independent Laboratory Validation of Prothioconazole-desthio in Different Matrices of Animal Origin Company Report No S21-08868 GLP Unpublished	N	UPL
KCP 5.2/04	Hitchens, J.	2023	Title - Prothioconazole-desthio and hydroxy metabolites: Method Validation in Honey Company Report No QG/21/009 GLP	N	UPL

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCP 5.2/05	Wanger, J.	2023	Title - Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Honey Company Report No S21-08357 GLP Unpublished	N	UPL
KCP 5.2/06	Kaiser, M.	2022	Title - Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Soil Company Report No S21-08358 GLP Unpublished	N	UPL
KCP 5.2/07	Kaiser, M.	2022	Title - Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Water Company Report No S21-08359 GLP Unpublished	N	UPL
KCP 5.2/08	Jooß, S.	2023	Title - Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Water Company Report No S21-08869 GLP Unpublished	N	UPL
KCP 5.2/09	Boubakri, N.	2023	Title - Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Urine Company Report No S21-08361 GLP Unpublished	N	UPL

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1	Richter, S., Stanislawski, T.	2021	Validation of Syngenta method RAM 305/03 for the determinaiton of residues of azoxystrobin and its metabolites R230310 in crop matrices by LC-MS/MS Eurofins Agroscience Services EAG Laboratories Report No. S20-05043 Study No. VV-892169	N	AIR4 TF
KCP 5.2	Amic, S.	2012	Azoxystrobin – Validation of Analytical Method for the Determination of Azoxystrobin and its Metabolite R234886 in Water. Report No. S11-03538 Document No. VV-401211 , ICI5504_11490 Test Facility Eurofins - ADME Bioanalyses GLP Unpublished	N	Syngenta Crop Protection AG Evaluated by zRMS-PL in RR of Orondis Evo, Part B5, June 2023
KCP 5.2	Brown, D.	2019	Azoxystrobin – Independent Laboratory Validation of Analytical Method GRM057.01A for the Determination of Residues of Azoxystrobin and its Metabolite R234886 in Water Report No. RES-00193 Document No. VV-619234 , ICI5504_12452 Test Facility ResChem Analytical Limited GLP Unpublished	N	Syngenta Crop Protection AG Evaluated by zRMS-PL in RR of Orondis Evo, Part B5, June 2023
KCP 5.2	Gemrot, F.	2011	Azoxystrobin – Validation of analytical method RAM 399/01 for the determination of azoxystrobin, R230310 and R234886 in human whole blood. Report No. S10-03815 Document No. VV-398250 , ICI5504_11467 Test Facility Eurofins - ADME Bioanalyses GLP Unpublished	N	Syngenta Crop Protection AG

AIR4 TF - the Azoxystrobin AIR-4 Task Force

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for prothioconazole and azoxystrobin

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

Comments of zRMS:	The analytical method was successfully validated and is considered suitable for the determination of residues of prothioconazole-desthio in crops: oilseed rape - seed and forage; wheat - grain, straw, forage; barley - grain, straw, forage; field bean (dried); orange (whole) and cucumber with an LOQ of 0.005 mg/kg. Mean recoveries were in the range of 60 – 120% with relative standard deviations of $\leq 30\%$ for all matrices at each level (0.005 and 0.05 mg/kg). The method is acceptable.
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Reference:	KCP 5.1.2/01
Report	Prothioconazole-desthio: Method Validation in Crops, J. Pearson, 2022, Report No. QG/20/011
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Quantitative determination of prothioconazole-desthio content in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of prothioconazole-desthio were extracted from crops with acetonitrile and QuEChERS extraction salts, followed by centrifugation. An aliquot of the acetonitrile phase was then cleaned up using d-SPE followed by centrifugation. An aliquot was diluted two-fold with acetonitrile:water (80:20, v/v) for oilseed rape seed; wheat – grain and straw; barley – grain and straw; and field bean (dried) matrices. An aliquot was diluted ten-fold with acetonitrile:water (80:10, v/v) for cucumber, orange (whole), wheat forage, barley forage and oilseed rape forage matrices. A portion of the final extract was then taken for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions, except for stability samples where the quantitation transition only was monitored.

Instrument Conditions

Liquid Chromatography:

Column:	Kinetex 5u XB-C18 100A, 150 x 4.6 mm, 5 μm		
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287		
Column Oven Temperature (°C):	40		
Injection Volume * (μL):	10		
Flow Rate (μL/min):	1000		
Mobile Phase A:	0.1 % Formic acid in water		
Mobile Phase B:	0.1 % Formic acid in methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	40	60
	1.00	40	60
	5.00	10	90
	6.00	10	90
	6.10	0	100
	7.00	0	100
	7.10	40	60
	9.00	40	60
Flow divert:	Time (minutes)	Position	
	0.0	To waste	
	4.0	To mass spec	
	6.0 - End	To waste	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)		
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)		

* Alternative volumes are allowed, based on instrument performance

Mass Spectrometry:

MS/MS System	API 5500
Ionisation Mode:	ESI
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	5500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10
Declustering Potential (DP)** (V)	126

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass*	Q3 Mass*	Dwell Time (msec)	Collision Energy* (CE) (V)	Collision Exit Potential* (CXP) (V)	Declustering Potential (DP)	Expected Retention time (±0.5mins)
Prothioconazole-desthio	1#	312.1	70.1	100	47	12	126	5.4
	2#	312.1	125.0	100	49	6	126	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

* Minor modifications are allowed to optimise instrument performance.

Results and discussions

The analytical method for the determination of prothioconazole-desthio content in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (< 30% LOQ) in reagent blank and duplicate control samples, demonstrating good selectivity of the method.

Linearity

Calibration curves were obtained from a minimum of eight calibration solutions containing prothioconazole-desthio. The standard calibration range was 0.075 to 3.0 ng/mL for wheat – straw and

forage; barley – straw and forage; and oilseed rape forage matrices and 0.150 – 6.0 ng/mL for orange (whole), cucumber, wheat grain, barley grain, oilseed rape seed and field bean (dried) matrices (equivalent to 0.0015 – 0.06 mg/kg), covering a range from 30% of the LOQ to 20% above the highest fortification level. Matrix matched standards were prepared. Correlation coefficients $r \geq 0.995$ were achieved in the validation and extract stability batches using a linear regression with a weighting, demonstrating acceptable linearity. On visual inspection of a plot of residuals, the data was randomly distributed, demonstrating suitability of the chosen function.

Accuracy and Precision

For each matrix, average recoveries and the overall average recovery at the LOQ fortification level were all within the acceptance range of 60-120% (0.005 mg/kg) and average recoveries and the overall average recovery at the 10 x LOQ fortification level were all within the acceptance range of 70-120% (0.05 mg/kg), demonstrating satisfactory accuracy. The relative standard deviation (RSD) at and the overall RSD did not exceed 30% at the LOQ level or 20% at the 10 x LOQ level, demonstrating satisfactory precision. In addition, LC-MS/MS monitoring two ion mass transitions is considered to be a highly specific technique.

Matrix Effects

Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions prepared at the same concentration. Experiments assessed whether or not matrix effects were significant (i.e. $\geq 20\%$ enhancement or suppression). No significant matrix effects were observed for orange, cucumber, field bean (dried), oilseed rape – seed and forage; wheat forage or barley – forage and straw; matrices. Significant matrix effects were observed for wheat – grain and straw; and barley grain matrices.

Stability of Standards and Extracts

Prothioconazole-desthio was shown to be stable in wheat forage, barley straw and oilseed rape forage sample extracts for 3 days, in cucumber sample extracts for 4 days, in wheat straw, barley forage and oilseed rape seed matrices for 5 days, in orange (whole) and field bean (dried) sample extracts for 6 days and in wheat grain and barley grain sample extracts for 7 days when stored refrigerated. All samples were analysed within the defined stability periods. Stock standard solutions of prothioconazole-desthio prepared in acetonitrile were shown to be stable for up to 86 days when stored refrigerated. Intermediate standard solutions of prothioconazole-desthio prepared in acetonitrile:water (9:1, v/v) were shown to be stable for up to 71 days when stored refrigerated.

LOD and LOQ

The limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) for prothioconazole-desthio in crops, equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole-desthio in crops, as confirmed by recovery efficiency testing.

Prothioconazole-desthio accuracy and precision data in wheat grain

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	104	105	108	106	100	5	104	2.8	100-108
0.05	103	102	105	99	101	5	102	2.1	99-105
Overall						10	103	2.6	99-108
Confirmatory Transition 312.1→125.0 m/z									
0.005	106	100	103	105	109	5	104	3.4	100-109
0.05	104	103	105	100	103	5	103	2.0	100-105
Overall						10	104	2.7	100-109

Prothioconazole-desthio accuracy and precision data in wheat forage

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	100	101	107	104	99	5	102	3.2	99-107
0.05	102	92	87	95	95	5	94	6.0	87-102
Overall						10	98	6.1	87-107
Confirmatory Transition 312.1→125.0 m/z									
0.005	101	102	105	104	101	5	102	1.7	101-105
0.05	104	94	88	96	95	5	95	5.8	88-104
Overall						10	99	5.4	88-105

Prothioconazole-desthio accuracy and precision data in wheat straw

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	94	117	97	108	97	5	103	9.4	94-117
0.05	110	108	92	99	119	5	106	9.8	92-119
Overall						10	104	9.2	92-119
Confirmatory Transition 312.1→125.0 m/z									
0.005	96	126	111	124	103	5	112	11.6	96-126
0.05	108	110	90	99	119	5	105	10.3	90-119
Overall						10	109	10.9	90-126

Table A 1: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in crops

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (> 30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points
Calibration range	0.075 to 3.0 ng/mL for wheat – straw and forage 0.150 – 6.0 ng/mL for wheat - grain equivalent to 0.0015 – 0.06 mg/kg
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD: 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The analytical method was successfully validated and is considered suitable for the determination of residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops: oilseed rape seeds, field beans (dried), barley grain, barley forage, cucumber, orange, oilseed rape forage, wheat forage, wheat straw, wheat grains and barley straw with an LOQ of 0.005 mg/kg in accordance with
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	SANTE/2020/12830 rev. 1. The method is acceptable.
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Reference: KCP 5.1.2/02

Report Hydroxy Metabolites of prothioconazole-desthio: Method Validation in Crops, D. Giancola, 2023, Report No. QG/22/001

Guideline(s): Yes - SANTE/2020/12830 rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4- hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

For all matrices, the extracts were evaporated to the aqueous remainder by rotary evaporation. The extracts were then adjusted to pH 3-4 using 1M HCl and incubated for hydrolysis at 80°C for one hour. After hydrolysis, the sample extracts were then adjusted to pH 7 using 1M sodium bicarbonate solution. Sample extracts were then cleaned up by liquid/liquid partition using cyclohexane/ethyl acetate (85:15, v/v). The organic layer was then evaporated to dryness under a stream of nitrogen. After reconstitution in acetonitrile and water, a portion of the final extract is taken for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS) monitoring two ion mass transitions.

Instrument Conditions

Liquid Chromatography:

Column:	Kromasil 5 µm C18 100A 4.6 × 250 mm			
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287			
Column Oven Temperature (°C):	40			
Injection Volume * (µL):	20			
Flow Rate (µL/min):	1000			
Mobile Phase A:	0.1 % Formic acid in water			
Mobile Phase B:	Methanol			
Gradient:	Time (minutes)	Flow Rate (µL/min)	% Mobile Phase A	% Mobile Phase B
	0.00	1000	60	40
	0.20	1000	33	67
	8.70	1000	33	67
	8.80	900	33	67
	10.30	900	5	95
	11.80	900	5	95
	12.00	900	60	40
	12.50	900	60	40
	13.50	900	60	40
Flow divert:	Time (minutes)		Position	
	0.00		To waste (B)	
	5.20		To mass spec (A)	
	9.70		To waste (B)	
	12.50		To mass spec (A)	
	13.40 to end		To waste (B)	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)			
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)			

* Alternative volumes are allowed, based on instrument performance

Mass Spectrometry:

MS/MS System	API 5500
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	3500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass ^A	Q3 Mass ^A	Dwell Time (msec)	Collision Energy ^A (CE)	Collision Exit Potential ^A (CXP)	Declustering Potential ^A (DP)	Expected Retention time (±0.5mins)
Prothioconazole- α -hydroxy-desthio	1#	328.265	69.900	100	36.00	10.00	80.00	5.76 and 7.85
	2#	328.265	141.200	100	39.00	10.00	80.00	
Prothioconazole-3-hydroxy-desthio	1#	328.142	70.000	100	37.00	10.00	46.00	7.29
	2#	328.142	141.00	100	47.00	10.00	46.00	
Prothioconazole-4-hydroxy-desthio	1#	328.154	70.000	100	35.00	12.00	121.00	8.35
	2#	328.154	141.100	100	53.00	10.00	121.00	
Prothioconazole-5-hydroxy-desthio	1#	327.948	70.000	100	33.00	14.00	151.00	8.94
	2#	327.948	141.000	100	45.00	22.00	151.00	
Prothioconazole-6-hydroxy-desthio	1#	327.899	140.872	100	68.00	10.00	69.00	12.9
	2#	327.899	69.824	100	44.00	10.00	69.00	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

^A Minor modifications are allowed to optimise instrument performance.

Results and discussions

The analytical method for the determination of hydroxy metabolites of prothioconazole-desthio content in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating the selectivity of the method.

Linearity

Calibration curves were obtained from a minimum of eight calibration solutions containing prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio. The standard calibration range was 0.375 to 15.0 ng/mL for oilseed rape seed, field beans (dried), barley grain, barley forage, cucumber, orange, barley straw and wheat straw matrices and 0.075 – 3.0 ng/mL for oilseed rape forage, wheat forage and wheat grain (equivalent to 0.0015 – 0.06 mg/kg), covering a range from 30% of the LOQ to 20% above the highest fortification level. Matrix matched standards were prepared. Correlation coefficients $r \geq 0.995$ were achieved in the validation and extract stability batches using a linear regression or quadratic regression with a weighting. On visual inspection of a plot of relative residuals, the data was randomly distributed, demonstrating suitability of the chosen function.

Accuracy and Precision

For each matrix, average recoveries and the overall average recovery at the LOQ fortification level were all within the acceptance range of 60-120% (0.005 mg/kg) and average recoveries and the overall average recovery at the 10 x LOQ fortification level were all within the acceptance range of 70-120% (0.05 mg/kg), demonstrating satisfactory accuracy. The relative standard deviation (RSD) at and the overall RSD did not exceed 30% at the LOQ level or 20% at the 10 x LOQ level, demonstrating satisfactory precision. In addition, LC-MS/MS monitoring two ion mass transitions is considered to be a highly specific technique.

Satisfactory accuracy and precision data were achieved for both ion mass transitions monitored, demonstrating that either transition may be used for quantification and/or confirmation of residues.

Matrix Effects

Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). No significant matrix effects were observed except for:

- prothioconazole-6-hydroxy-desthio in barley forage, barley straw, wheat straw, and oilseed rape seed.
- prothioconazole-5-hydroxy-desthio in oilseed rape seed and prothioconazole-5- hydroxy-desthio (quantitation transition) in oilseed rape forage.
- prothioconazole-4-hydroxy-desthio (confirmatory transition) in field bean (dried) and prothioconazole-4-hydroxy-desthio (quantitation transition) in oilseed rape forage
- prothioconazole-3-hydroxy-desthio in oilseed rape forage

Wheat grain matrix effect experiment was performed in two different analytical runs. Data obtained for prothioconazole-5-hydroxy-desthio (confirmatory transition) show contradictory results and no final matrix effect assessment can be determined. For this reason, it is recommended to use matrix matched standards when analysing prothioconazole-5-hydroxy-desthio (confirmatory transition) in wheat grain.

Wheat straw matrix effect experiment was performed in two different analytical runs. Data obtained for prothioconazole-5-hydroxy-desthio show contradictory results and no final matrix effect assessment can be determined. For this reason, it is recommended to use matrix matched standards when analysing prothioconazole-5-hydroxy-desthio in wheat straw.

Stability of Standards and Extracts

Prothioconazole- α -hydroxy-desthio, Prothioconazole-3-hydroxy-desthio, Prothioconazole-4- hydroxy-desthio, Prothioconazole-5-hydroxy-desthio and Prothioconazole-6-hydroxy-desthio were shown to be stable in wheat forage sample extracts for 8 days; in field bean (dried) and barley grain sample extracts for 7 days; in oilseed rape forage for 6 days; in wheat straw, barley straw, orange and wheat grain sample extracts for 5 days when stored refrigerated.

Standard solution stability for prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio, prothioconazole-6-hydroxy-desthio and prothioconazole- α -hydroxy-desthio prepared in acetonitrile:water 80:20 (v/v) was established in study QG/21/009 for 54 days when stored at approximately 4°C.

LOD and LOQ

The limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) for prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops, equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops, as confirmed by recovery efficiency testing.

Table A 2: Characteristics for the analytical method used for validation of hydroxy metabolites of prothioconazole-desthio residues in crops

	Hydroxy metabolites of prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points.
Calibration range	0.375 - 15.0 ng/mL for oilseed rape seed, field beans (dried), barley grain, barley forage, cucumber, orange, barley straw and wheat straw matrices and 0.075 – 3.0 ng/mL for oilseed rape forage, wheat forage and wheat grain (equivalent to 0.0015 – 0.06 mg/kg). $r \geq 0.995$
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	LOQ = 0.005 mg/kg LOD = 0.0015 mg/kg

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The analytical method was successfully validated and is considered suitable for the determination of residues of 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in crops: barley grain, barley plant, barley straw, cucumber, field bean, oilseed rape plant, oilseed rape seed, orange (whole), wheat grain, wheat plant and wheat straw in accordance with SANTE/2020/12830 rev. 1. The method is acceptable.
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Reference: KCP 5.1.2/03

Report Triazole Derived Metabolites (TDM's) (1,2,4-triazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops, B. Phipps, 2024, Report No. QG/20/012

Guideline(s): Yes - SANTE/2020/12830 rev. 1

Deviations: Yes – please refer to the report for a complete list. In summary, the deviations are considered not to have an impact on the validity of the study.

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of residues of triazole derived metabolites (Triazole alanine (TA) Triazole acetic acid (TAA), Triazole lactic acid (TLA) and 1,2,4-triazole (TRZ)) in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of TA, TAA, TLA and TRZ were extracted from barley grain, barley plant, barley straw, cucumber, field bean, oilseed rape plant, oilseed rape seed, orange (whole), wheat grain, wheat plant and wheat straw with methanol: water (4:1 v/v) by homogenisation with ultra-turrax followed by filtration under vacuum through celite. This was followed by filtration through SPE, concentration and reconstitution in water. A portion of the final extract was then filtered, diluted and internal standard added.

Final determination was by liquid chromatography with tandem mass spectrometry (LC-MS/MS) with SelexION technology, monitoring two ion mass transitions (or a single ion transition but with separate HPLC conditions)*, except for stability samples where the quantitation transition only was monitored.

The method was validated in terms of linearity (calibration), specificity (selectivity), accuracy (recovery), precision (repeatability), matrix effects and stability.

*The exception to this was for triazole alanine where three sets of ion data were generated for cucumber, barley grain, barley plant, barley straw, wheat grain, wheat plant, wheat straw. Any valid data generated for any of these 3 ions is reported.

Instrument Conditions

Liquid Chromatography:

Column:	Thermo Aquasil C18, 150 × 3.0mm, 3µm		
Guard Column:	C18, 4 x 3.0mm ID		
Column Oven Temperature (°C):	40		
Injection Volume * (µL):	20		
Flow Rate (µL/min):	1000		
Mobile Phase A:	0.2 % Acetic acid in water		
Mobile Phase B:	0.2 % Acetic acid in Methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	100	0
	2.00	100	0
	5.00	10	90
	5.50	10	90
	5.60	100	0
	7.00	100	0
Flow divert:	Time (minutes)	Position	
	0.0	To waste (A)	
	0.1	To mass spec (B)	
	3.0 – 7.0	To waste (A)	
Autosampler Wash Solvent 1 (Strong):	Methanol		
Autosampler Wash Solvent 2 (Weak):	Methanol:water (10:90 v/v)		
Autosampler wash method:	Post clean with solvent 1 (s):	2	
	Valve clean with solvent 1 (s):	3	
	Valve clean with solvent 2 (s):	3	

* Alternative volumes are allowed, based on instrument performance.

Mass Spectrometry:

DMS-MS/MS System:	API 6500 with SelexION
Ionisation Mode:	ESI
Ion Source:	Turbo Spray
Polarity:	Positive
Scan Type:	MRM
Ion Spray Voltage (IS) (V)**:	5500
Collision Gas (CAD)**:	Medium (-2.00)
Source Temperature (TEM) (°C)**:	650
DMS Temperature (DT) (°C):	150 (low)
Curtain Gas (CUR)**:	50.0
Ion Source Gas 1 (GS1)**:	40
Ion Source Gas 2 (GS2)**:	60
Entrance Potential (EP) (V):	10
Separation voltage (SV) V:	3000

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition #	Q1 Mass	Q3 Mass	Dwell Time (msec)	CE (V)*	CXP (V)*	DP*	COV*	DMO*
TA	1	157.0	70.0	100	19.00	6.00	6.00	-0.83	-9.95
TA_ISTD	1	162.0	75.0	100	17.00	10.00	66.00	0.00	0.97

* Minor modifications are allowed to optimise instrument performance.

Transition 1 (confirmatory) is to be used for sample quantification for all non IL-IS transitions.

Results and discussions

The analytical method for the determination of residues of triazole derived metabolites (Triazole alanine (TA) Triazole acetic acid (TAA), Triazole lactic acid (TLA) and 1,2,4-triazole (TRZ)) in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences were detected (>30% of the limit of quantitation) in the replicate control samples for some analytes and matrices, please refer to the study for further details. Where interferences >30% of the LOQ in the control samples were observed, the average control found value in mg/kg was subtracted from the recovery value in mg/kg. All reagent blank samples analysed were <30% LOQ. Selectivity was confirmed by the use of LC-MS/MS monitoring two ion mass transitions or a single ion mass transition using two significantly different HPLC columns. A minimum of one reagent blank and two untreated replicates were analysed within each set of validation data (for each matrix and analyte).

Linearity

Calibration curves were obtained from a minimum of five calibration solutions containing the relevant analytes for analysis. Mixed analyte standards were used where necessary. The standard calibration range was 0.3 to 50 ng/mL, covering a range from at least 30% of the LOQ to at least 20% above the highest concentration level.

Accuracy and Precision

Average recoveries and the RSD calculated at each fortification level were within the limits described in the table below; demonstrating satisfactory accuracy and precision.

Concentration level (mg/kg)	Range of mean recoveries (%)	Precision, RSD (%)
≤0.01	60-120	30
>0.01 - ≤ 0.1	70-120	20
>0.1 - ≤ 1.0	70-110	15
>1	70-110	10

Satisfactory accuracy and precision data were achieved for both ion mass transitions monitored (or single ion transitions via two HPLC columns), demonstrating that either transition may be used for quantitation and/or confirmation of residues.

Matrix Effects

Matrix effects were investigated comparing peak area and peak area ratios of solvent standard solutions to peak area and peak area ratios of matrix-matched standard solutions prepared at the same concentration. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). Matrix effects were investigated with and without the addition of internal standard. Varying degrees of matrix effects were observed without the presence of internal standard. No significant matrix effects were observed. (i.e. >20% enhancement or suppression) when the internal standard was present. Matrix effects were negated by the addition of internal standard as such solvent standards, which were used for all analysis.

Stability of Solutions

Stock solutions of each analyte prepared in solvent were stored in a refrigerator. After storage, an aliquot of the stock solutions were diluted and analysed against a freshly prepared standard. Working, intermediate and calibration solutions were also stored and tested for stability. Peak areas of the stored solutions were compared to freshly prepared solutions and they were deemed stable if the areas were within 10% of each other.

LOD and LOQ

The limit of detection (LOD) was established at 30% LOQ for each analyte and matrix.

Table A 3: Characteristics for the analytical method used for validation of triazole derived metabolites residues in wheat

	TRZ	TAA	TA	TLA
Specificity	samples for some analytes and matrices, please refer to the study for further details. Where interferences >30% of the LOQ in the control samples were observed, the average control found value in mg/kg was subtracted from the recovery value in mg/kg.			
Calibration (type, number of data points)	Minimum of 5 calibration points.			
Calibration range	0.3 – 50 ng/mL (equivalent to 0.0012 – 0.2 mg/kg) r ≥ 0.995	0.3 – 50 ng/mL (equivalent to 0.0024 – 0.4 mg/kg) r ≥ 0.995	0.3 – 50 ng/mL (equivalent to 0.0024 – 0.4 mg/kg) r ≥ 0.995	0.3 – 50 ng/mL (equivalent to 0.0012 – 0.2 mg/kg) r ≥ 0.995
Assessment of matrix effects is presented	Yes			
Limit of determination/quantification	LOQ = 0.005 mg/kg (wheat grain, wheat plant and wheat straw) LOD = 0.0015 mg/kg (wheat grain, wheat plant and wheat straw)	LOQ = 0.01 mg/kg (wheat grain), 0.02 mg/kg (wheat plant) and 0.05 mg/kg (wheat straw) LOD = 0.003 mg/kg (wheat grain), 0.006 mg/kg (wheat plant) and 0.015 mg/kg (wheat straw)	LOQ = 0.01 mg/kg (wheat grain), 0.02 mg/kg (wheat plant) and 0.05 mg/kg (wheat straw) LOD = 0.003 mg/kg (wheat grain), 0.006 mg/kg (wheat plant) and 0.015 mg/kg (wheat straw)	LOQ = 0.005 mg/kg (wheat grain) and 0.05 mg/kg (wheat plant and wheat straw) LOD = 0.0015 mg/kg (wheat grain) and 0.0015 mg/kg (wheat plant and wheat straw)

Conclusion

The validation criteria for the method used to determine triazole derived metabolites residues in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The multi-residue method QuEChERS was successfully validated and is considered suitable for the determination of azoxystrobin and its metabolite z-azoxystrobin in/on oilseed rape whole plant (high water content) and seeds (high oil content) matrices with
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	LOQ of 0.01 mg/kg in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment and monitoring.
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Reference:	KCP 5.1.2/04
Report	Validation of the Analytical Method for Determination of Azoxystrobin in OSR WholePlant and OSR Seeds; Rastogi T., 2024, Report No. UPL/2023/1991
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Quantitative determination of prothioconazole metabolites in oilseed rape matrices determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1. The methodology is based on the Multi-residue method QuEChERS.

samples of investigated matrices, after addition of water, were extracted with acetonitrile. The samples were shaken sing horizontal shaker. A Supel QuE Citrate Tube (55227-U) containing mixture of citrate salts, magnesium sulphate and sodium chloride was added. The extracts were shaken and centrifuged. The samples were in acetonitrile/water (1/1, v/v) prior to the LC-MS/MS analysis for determination of both analytes.

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 404>344 and m/z 404>372 for both analytes). Due to enhanced specificity mass transition m/z 404>344 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

Example Instrument Conditions

Liquid Chromatography and Mass Spectrometry:

HPLC system	Agilent Infinity 1290 Series HPLC system (vacuum solvent degasser, binary HPLC pump, Multisampler, Multicolumn Thermostat)					
Pre-column	Phenomenex C ₁₈ , 4x3 mm, Art. No. AJO-8762					
Column	Kromasil C18 (50 mm x 3.0 mm, 5.0 µm, Kromasil, Art. No. M05CLC05)					
Column oven temperature	40 °C					
Injection volume	30 µL					
Mobile phases	Eluent A: Water + 0.2 % acetic acid Eluent B: Acetonitrile					
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]		
	0.0	50	50	1000		
	2.5	50	50	1000		
Divert valve	Not used					
Retention times	z-Azoxystrobin: approx. 1.1 min; Azoxystrobin: approx. 1.4 min					
Mass spectrometric conditions						
MS system	SCIEX 4000 Triple Quadrupole MS/MS System, SCIEX*					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)	Ionspray turbo heater (TEM)		550 °C		
Curtain gas (CUR)	Nitrogen set at 20 (arbitrary units)	Gas flow 1 (GS1)		Nitrogen set at 40 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 5 (arbitrary units)	Gas flow 2 (GS2)		Nitrogen set at 70 (arbitrary units)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Azoxystrobin	404 → 344* (pos)	56	10	35	12	200
	404 → 372 (pos)	56	10	21	12	200
z-Azoxystrobin	404 → 344* (pos)	56	10	35	12	200
	404 → 372 (pos)	56	10	21	12	200

Proposed for quantification. Both of the mass transitions listed can be used for quantification.

*Measurements (for stability testing only) were also taken with a Sciex 5500 Triple Quad QTrap MS/MS system and a Sciex 6500+ Triple Quad MS/MS system. For this purpose, only parameters that are device-specific and not related to the analytes were adjusted.

Results and discussions

The analytical method for the determination of prothioconazole metabolites in oilseed rape matrices has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole metabolites were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating the selectivity of the method.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.15 ng/mL to 15 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any diluted sample extract. The calibration curve does not exceed two (2) orders of magnitude.

Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass

transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, correlation coefficients (R) were ≥ 0.99 . Representative regression curves showed no discernible pattern.

Accuracy and Precision

Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system. All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2. The results are summarised in the following table:

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Azoxystrobin							
Mass Transition m/z 404 → 344 (Quantification)							
Oilseed rape whole plant	0.01	107, 107, 104, 103, 101	104	2.6	5	102	3.5
	0.1	102, 96.4, 101, 103, 97.2	99.9	3.0	5		
Oilseed rape seeds	0.01	95.4, 85.4, 87.2, 88.4, 86.2	88.5	4.5	5	94.8	7.7
	0.1	100, 103, 104, 99.2, 99.8	101	2.0	5		
Mass Transition m/z 404 → 372 (Confirmation)							
Oilseed rape whole plant	0.01	113, 113, 107, 99.4, 98.0	106	6.8	5	104	5.7
	0.1	104, 98.6, 100, 105, 98.0	101	3.1	5		
Oilseed rape seeds	0.01	97.6, 81.8, 94.6, 94.2, 77.0	89.0	10	5	93.9	8.5
	0.1	100, 98.8, 101, 96.6, 96.8	98.7	2.0	5		
z-Azoxystrobin							
Mass Transition m/z 404 → 344 (Quantification)							
Oilseed rape whole plant	0.01	107, 108, 96.4, 94.6, 96.0	100	6.4	5	101	4.5
	0.1	103, 99.6, 104, 98.6, 101	101	2.2	5		
Oilseed rape seeds	0.01	96.6, 93.0, 106, 99.4, 81.2	95.2	9.6	5	103	9.8
	0.1	114, 109, 110, 110, 107	110	2.3	5		
Mass Transition m/z 404 → 372 (Confirmation)							
Oilseed rape whole plant	0.01	103, 103, 99.4, 94.2, 92.2	98.4	5.2	5	99.3	3.8
	0.1	102, 99.8, 102, 97.2, 101	100	1.9	5		
Oilseed rape	0.01	98.6, 97.2, 107, 100, 87.4	98.0	7.1	5	104	8.0

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
seeds	0.1	115, 110, 111, 110, 106	110	2.8	5		

Matrix Effects

Not required. Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with solvent standard solutions.

Stability of Standards and Extracts

Following the first analysis, the final extracts of samples fortified at the 10x LOQ level together with one control sample extract were stored at typically 1 °C to 10 °C in the dark. After storage the final extracts were analysed against freshly prepared calibration standards. One (1) mass transition per analyte was evaluated. The mean recovery values of the stored extracts were in the range of 70 % - 120 % with a relative standard deviation ≤ 20 % when analysed against freshly prepared calibration standards. Therefore, extracts are considered to be stable when stored at 1 °C to 10 °C for at least 7 days in the dark.

Storage stability testing with fortified or incurred samples was not conducted as part of this study.

Conclusion

The method was successfully validated for the determination of Azoxystrobin and z-Azoxystrobin in/on oilseed rape whole plant and oilseed rape seeds from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in feeding solution with LOQ of 0.35 g/L in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment.
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Reference: KCP 5.1.2/05

Report Azoxystrobin 160 + Prothioconazole 200 g/L SC: Chronic Oral Toxicity Test on the Honey Bee (*Apis mellifera* L.) in the Laboratory; Horstmann, C. 2024; Study No.: UPL/2023/1657

Guideline(s): Yes - SANTE/2020/12830 rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range. Samples were diluted while solutions were stirring, if necessary. The samples were analysed on the day of processing (therefore stability testing was not required).

Fortification Procedure:

The test item was dissolved in matrix (50 % w/v sucrose solution + 0.1 % xanthan) which was sonicated for 5 minutes and stirred for 60 minutes to obtain fortified samples of 22 g test item/L. The test item was dissolved in matrix (sonicated for 5 minutes and stirred for 60 minutes) to obtain fortification stock solutions of 3.5 g test item/L. Appropriate amounts of these stock solutions were diluted in matrix to obtain fortified samples at 0.35 g test item/L. Fortified samples were diluted with solvent mixture to match the calibration range.

Samples were analysed using HPLC-UV-method (250 nm)

Chromatographic Conditions

HPLC-Conditions:

System: Waters Acquity
Software: Empower 3
Column: Kinetex C18 (50 × 2.1 mm; 2.6 µm)
Mobile Phase: A: acetonitrile + 0.1 % formic acid B: pure water + 0.1 % formic acid
Gradient Mode:

Time [min] initial
Solvent A [%] 40
Solvent B [%] 60

Total Run Time: 6.0 minutes

Flow Rate: 0.4 mL/min

Temperature: 25 °C

Injection Volume: 2 µL

Detector: DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm

Retention Time: Azoxystrobin: 2.8 minutes Prothioconazole: 4.2 minutes

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Calibration Range: 0.5 to 20 mg Azoxystrobin/L corresponding to 20% of LOQ to 157% of higher fortification level ($y = 12424x - 265$, $r = 1.0000$), and 0.5 to 20 mg Prothioconazole/L corresponding to 16% of LOQ to 126 % of higher fortification level ($y = 10643x - 1320$, $r = 0.9999$). For each calibration plot, 7 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effect were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

Matrix matched standards were used for the determination of the active ingredients.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) $\leq 20\%$ was obtained.

Azoxystrobin = 0.35 g/L

Prothioconazole = 0.35 g/L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 0.5 mg/L

Prothioconazole = 0.5 mg/L

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples (50% w/v sucrose solution + 0.1% xanthan) and subsequent determination of the recoveries upon applying the test method. Acceptable recoveries were obtained, as shown in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
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Azoxystrobin	0.35 g test item/L	93, 90, 95, 93, 92	93	2
Azoxystrobin	22 g test item/L	100, 96, 99, 105, 98	100	4
Azoxystrobin	Oral feeding solution (384 mg test item/kg)	95	-	-
Azoxystrobin	Oral feeding solution (15000 mg test item/kg)	99	-	-
Prothioconazole	0.35 g test item/L	88, 84, 91, 88, 88	88	3
Prothioconazole	22 g test item/L	98, 93, 96, 102, 95	97	3
Prothioconazole	Oral feeding solution (384 mg test item/kg)	92	-	-
Prothioconazole	Oral feeding solution (15000 mg test item/kg)	97	-	-

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the oral feeding solution fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the oral feeding and contact solutions in accordance to guidance document SANTE/2020/12830, rev.1 for risk assessment. The LOQs were: For azoxystrobin and prothioconazole of 5 g/L (contact) For azoxystrobin and prothioconazole of 0.6 g/L (oral).
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Reference:	KCP 5.1.2/06
Report	Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects (Acute Contact and Oral) on Honey Bees (<i>Apis mellifera</i> L.) in the Laboratory; Horstmann, C. 2024; Study No.: UPL/2023/1780
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range. Samples were diluted while solutions were stirring, if necessary. The samples were analysed on the day of processing.

Fortification Procedure:

Contact Test:

The test item was dissolved in matrix contact (pure water + 0.1 % v/v Triton X-100) which was stirred for 60 minutes to get fortified samples of about 5 g test item/L and 100 g test item /L. Fortified samples were diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range.

Oral Test:

The test item was dissolved in matrix oral (50 % w/v sucrose solution) which was sonicated for 10 minutes stirred for 60 minutes to get fortified samples of about 30 g test item/L. Aliquots of these solutions were further diluted with matrix oral to obtain fortified samples of about 0.6 g test item /L. Fortified samples were diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range.

Samples were analysed using HPLC-UV-method (250 nm)

Chromatographic Conditions

HPLC-Conditions:

System: Waters Acquity
Software: Empower 3
Column: Kinetex C18 (50 × 2.1 mm; 2.6 µm)
Mobile Phase: A: acetonitrile + 0.1 % formic acid B: pure water + 0.1 % formic acid
Gradient Mode:
Time [min] 0-6
Solvent A [%] 40
Solvent B [%] 60

Total Run Time: 6.0 minutes

Flow Rate: 0.4 mL/min

Temperature: 25 °C

Injection Volume: 2 µL

Detector: DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm

Retention Time: Azoxystrobin: 2.7 minutes Prothioconazole: 4.1 minutes

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Contact test:

Calibration Range: 2 to 25 mg Azoxystrobin/L corresponding to 28% of LOQ to 215% of higher fortification level ($y = 12494x - 1036$, $r = 1.0000$), and 2 to 25 mg Prothioconazole/L corresponding to 22% of LOQ to 174% of higher fortification level ($y = 10934x - 3194$, $r = 1.0000$). For each calibration plot, 7 calibration standards were used. Samples were diluted to fit within the calibration range.

Oral test:

Calibration Range: 2 to 30 mg Azoxystrobin/L corresponding to 23% of LOQ to 172% of higher fortification level ($y = 12562x + 3307$, $r = 0.9993$), and 2 to 30 mg Prothioconazole/L corresponding to 19% of LOQ to 139% of higher fortification level ($y = 10699x + 695$, $r = 0.9993$). For each calibration plot, 7 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effect were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

Matrix matched standards were used for the determination of the active ingredients.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120% of nominal) with a relative standard deviation (RSD) $\leq 20\%$ was obtained.

Azoxystrobin = 5 g/L (contact)

Prothioconazole = 5 g/L (contact)

Azoxystrobin = 0.6 g/L (oral)

Prothioconazole = 0.6 g/L (oral)

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 2 mg/L (contact and oral)

Prothioconazole = 2 mg/L (contact and oral)

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples and subsequent determination of the recoveries upon applying the test method. Acceptable recoveries were obtained, as shown in the following table:

Test	Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Contact	Azoxystrobin	5 g test item/L	106, 105, 107, 110, 106	107	2
Contact	Azoxystrobin	100 g test item/L	105, 103, 107, 107, 105	105	2
Contact	Azoxystrobin	Contact application solution A (400 µg product/bee)	99	-	-
Contact	Azoxystrobin	Contact application solution B(400 µg product/bee)	99	-	-
Contact	Prothioconazole	5 g test item/L	101, 100, 102, 104, 102	102	1
Contact	Prothioconazole	100 g test item/L	100, 98, 101, 102, 100	100	1
Contact	Prothioconazole	Contact application solution A (400 µg product/bee)	94	-	-
Contact	Prothioconazole	Contact application solution B(400 µg product/bee)	93	-	-
Oral	Azoxystrobin	0.6 g test item/L	99, 101, 100, 97, 98	99	2
Oral	Azoxystrobin	30 g test item/L	102, 104, 106, 101, 105	104	2
Oral	Azoxystrobin	Application solution (400 µg product/bee)	121	-	-
Oral	Prothioconazole	0.6 g test item/L	99, 100, 100, 96, 98	99	2
Oral	Prothioconazole	30 g test item/L	102, 104, 105, 101, 105	103	2
Oral	Prothioconazole	Application solution (400 µg product/bee)	120	-	-

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the oral feeding and contact solutions fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the oral feeding and contact solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQs were: For azoxystrobin and prothioconazole of 6.4 g/L (contact) For azoxystrobin and prothioconazole of 0.5 g/L (oral).
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Reference: KCP 5.1.2/07

Report	Azoxystrobin 160 g/L + Prothioconazole 200 g/L SC: Acute Contact and Oral Toxicity to Bumblebees (<i>Bombus terrestris</i> L.) in the Laboratory; Horstmann, C. 2024; Study No.: UPL/2023/2345
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range. Samples were diluted while solutions were stirring, if necessary.

Fortification Procedure:

Contact Test:

The test item was dissolved in matrix contact (pure water+ 0.1 % v/v Triton X-100) which was sonicated for 5 minutes and stirred for 60 minutes to get fortified samples of about 160 g test item/L. Aliquots of these solutions were diluted in matrix contact to obtain fortified samples at approximately 6.4 g test item/L.

Oral Test:

The test item was dissolved in matrix oral (50 % w/v sucrose solution) which was sonicated for 5 minutes and stirred for 60 minutes to get fortified samples of about 25 g test item/L. Aliquots of these solutions were diluted in matrix oral/contact to obtain fortified samples at approximately 0.5 g test item/L.

Fortified samples were diluted with solvent mixture (acetonitrile/pure water (50/50 v/v)) to match the calibration range.

Samples were analysed using HPLC-UV-method (250 nm)

Chromatographic Conditions

HPLC-Conditions:

System: Waters Acquity
Software: Empower 3
Column: Kinetex C18 (50 × 2.1 mm; 2.6 µm)
Mobile Phase: A: acetonitrile + 0.1 % formic acid B: pure water + 0.1 % formic acid
Total Run Time: 6.0 minutes
Flow Rate: 0.4 mL/min
Temperature: 25 °C
Injection Volume: 2 µL
Detector: DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Contact test:

Calibration Range: 2 to 30 mg Azoxystrobin/L corresponding to 22 % of LOQ to 162 % of higher fortification level ($y = 12811x - 1063$, $r = 0.9998$), and 2 to 30 mg Prothioconazole/L corresponding to 17 % of LOQ to 131 % of higher fortification level ($y = 10784x - 1979$, $r = 0.9998$). For each calibration plot, 8 calibration standards were used. Samples were diluted to fit within the calibration range.

Oral test:

Calibration Range: 2 to 30 mg Azoxystrobin/L corresponding to 28 % of LOQ to 166 % of higher fortification level ($y = 12672 x - 1371$, $r = 1.0000$), and 2 to 30 mg Prothioconazole/L corresponding to 22 % of LOQ to 134 % of higher fortification level ($y = 10930 x - 2753$, $r = 1.0000$). For each calibration plot, 8 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effect were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect (≥ 20 %) was observed.

Matrix matched standards were used for the determination of the active ingredients.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) ≤ 20 % was obtained.

Azoxystrobin = 6.4 g/L (contact)

Prothioconazole = 6.4 g/L (contact)

Azoxystrobin = 0.5 g/L (oral)

Prothioconazole = 0.5 g/L (oral)

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 2 mg/L (contact and oral)

Prothioconazole = 2 mg/L (contact and oral)

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples and subsequent determination of the recoveries upon applying the test method. The recovery results obtained are provided in the following table:

Test	Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Contact	Azoxystrobin	6.4 g test item/L	103, 101, 39 (outlier), 103, 103, 107, 106, 106	104	2
Contact	Azoxystrobin	160 g test item/L	102, 79, 84, 102, 89, 107, 105, 105	97	11
Contact	Azoxystrobin	contact application solution (8.12 g product/L) - A	63	-	-
Contact	Azoxystrobin	contact application solution (8.12 g product/L) - A (replicate to confirm result)	63	-	-
Contact	Azoxystrobin	contact application solution (8.12 g product/L) - B	94	-	-
Contact	Azoxystrobin	contact application solution (130 g product/L) - A	93	-	-
Contact	Prothioconazole	6.4 g test item/L	101, 100, 37 (outlier), 102, 101, 103, 102, 102	102	1
Contact	Prothioconazole	160 g test item/L	102, 79, 84, 102, 89, 104, 101, 102	95	10
Contact	Prothioconazole	contact application solution (8.12 g product/L) - A	53	-	-

Contact	Prothioconazole	contact application solution (8.12 g product/L) – A (replicate to confirm result)	52	-	-
Contact	Prothioconazole	contact application solution (8.12 g product/L) - B	84	-	-
Contact	Prothioconazole	contact application solution (130 g product/L) - A	93	-	-
Oral	Azoxystrobin	0.5 g test item/L	93, 99, 102, 98, 95	97	4
Oral	Azoxystrobin	25 g test item/L	99, 97, 98, 98, 100	98	1
Oral	Azoxystrobin	feeding solution (0.69 g product/kg)	97	-	-
Oral	Azoxystrobin	feeding solution (16.25 g product/kg)	100	-	-
Oral	Prothioconazole	0.5 g test item/L	90, 95, 99, 94, 91	94	1
Oral	Prothioconazole	25 g test item/L	96, 94, 96, 95, 96	95	1
Oral	Prothioconazole	feeding solution (0.69 g product/kg)	94	-	-
Oral	Prothioconazole	feeding solution (16.25 g product/kg)	97	-	-

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the oral feeding and contact solutions fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the feeding solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQs were: for azoxystrobin of 7.5 g/L, for Prothioconazole of 9.4 g/L.
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Reference: KCP 5.1.2/08

Report Effects of Azoxystrobin 160 + Prothioconazole 200 g/L SC on honeybees (*Apis mellifera* L.) 22-day larval toxicity test with repeated exposure; Venturi, S. 2024; Study No.: UPL/2023/2016

Guideline(s): Yes - SANTE/2020/12830 rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The HPLC-DAD analytical method for the determination of Azoxystrobin and Prothioconazole in water solution samples from the honeybee chronic larval test was validated according to SANTE/2020/12830, Rev. 2 (14/02/2023) guideline

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture to match the calibration range. Samples were diluted while solutions were stirring, if necessary.

Samples were analysed using HPLC-UV-method.

Equipment

Apparatus: Agilent HPLC with DAD detector, 1200 series with Chemstation
Data analysis SW: OpenLab CDS ChemStation Edition for LC & LC/MS System, Version C.01.08 (Agilent Technologies, 2001-2017)
Column: Phenomenex Kinetex C18 2.6 µm 100 A 3 x 50 mm
Pipettes: Gilson
Analytical balance: Mettler Toledo XP105DR
Fridge/Freezer: Liebherr KGT3925 CSE

Experimental conditions

Eluent A: water acidified with 0.1% phosphoric acid
Eluent B: acetonitrile
%Ratio A/B: 50/50
Flow: 0.500 mL/min
Injected volume: 2.00 µL
Column temperature: 35.0°C
Runtime: 5 minutes
Analysed wavelength: 210 nm for Azoxystrobin, 254 nm for Prothioconazole
Retention Time:
Approx. 1.6 min for Azoxystrobin
Approx. 2.1 min for Prothioconazole

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Calibration Range: 2.4833-124.1647 mg Azoxystrobin/L corresponding to 4.9666-4515.0807 mg Azoxystrobin/L in the undiluted samples ($y = 27.395100x - 4.225800$, $r = 1.0000$), and 3.0843-154.2170 mg Prothioconazole/L corresponding to 6.1687-5607.8913 mg Prothioconazole/L in the undiluted samples ($y = 8.498800x - 6.471000$, $r = 0.9999$). For each calibration plot, 5 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effect were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

On the base of matrix effect evaluation, the analysed solutions were prepared in diluent (acetonitrile/water 1:1).

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) $\leq 20\%$ was obtained.

Azoxystrobin = 7.5 g/L

Prothioconazole = 9.4 g/L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 2.48 mg/L

Prothioconazole = 3.08 mg/L

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples and subsequent determination of the recoveries upon applying the test method. The recovery results obtained are provided in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Azoxystrobin	77 mg/L	97.48, 98.93, 99.43, 99.02, 99.82	98.94	0.9
Azoxystrobin	7.5 mg/L	87.80, 94.06, 85.42, 83.10, 84.37	86.37	4.98
Prothioconazole	92 mg/L	96.23, 97.20, 97.93, 96.43, 97.56	97.07	0.75
Prothioconazole	9.4 mg/L	86.07, 91.94, 83.68, 83.49, 85.17	86.07	4.01

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the feeding solutions fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the test solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQs for azoxystrobin and for prothioconazole were 0.006 mg test item/L.
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Reference: KCP 5.1.2/09

Report Azoxystrobin 160g/L + Prothioconazole 200g/L SC: Toxicity to *Pseudokirchneriella subcapitata* in an Algal Growth Inhibition Test; Bauer, J. & Mollandin, G., 2024; Study No.: UPL/2024/0581

Guideline(s): Yes - SANTE/2020/12830 rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Samples were prepared by direct dilution of samples followed by analysis via LC-MS/MS.

Sample Preparation:

Biological treatment samples and control samples:

The samples were allowed to thaw to room temperature. They were then shaken well and treated with ultrasound for 1 - 2 minutes to obtain homogenous samples. The samples were already diluted with D1 solvent (acetonitrile/water/formic acid/cysteine hydrochloride, 400/600/2/0.2 (v/v/v/w)) by factor 1.25 directly after sampling. The samples were diluted further with solvent (test water (OECD Medium)/D1, 4/1, v/v) to match the calibration range, if necessary. The samples were centrifuged (13,000 rpm, 3 minutes) before analysis.

Fortified Samples:

Fortified samples were prepared on the day of analysis. Approximately 30 mg of the test item were homogeneously dispersed dissolved (1 - 2 minutes ultrasonication, 41 -120 minutes stirring) in 50 mL test water to obtain a stock solution of approximately 0.6 g test item/L. Exact weighing and purity of the test item was considered for results evaluation. Three independent stock solutions were prepared. Appropriate amounts of these stock solutions were diluted with test water to obtain fortified samples at a level of 0.006 and 12 mg test item/L.

The samples were shaken well and were diluted with D1 solvent (acetonitrile/water/formic acid/cysteine hydrochloride, 400/600/2/0.2 (v/v/v/w)) by factor 1.25. The samples were diluted further with solvent (test water (OECD Medium)/D1, 4/1, v/v) to match the calibration range, if necessary. The samples were centrifuged (13,000 rpm, 3 minutes) before analysis.

LC-MS/MS Conditions

LC: Agilent Series 1290 pump and autosampler

Column: Phenomenex Kinetex C18 100 Å, 50 * 2.1 mm, 2.6 µm

Temperature: 40°C

Mobile Phase:

- A: HPLC water containing 0.12 % formic acid and 10 mM ammonium formate
- B: Methanol / HPLC water (95/5, v/v) containing 0.12 % formic acid and 10 mM ammonium formate

Gradient:

Time (min)	%A	%B
0.0	70	30
5.0	0	100
6.0	0	100
6.1	70	30
10	70	30

Run Time: 10 min

Flow Rate: 0.4 mL/min

Injection Volume: 20 µL

Detector: Mass spectrometer API 5500

Parameters:

- Ion Source: Electrospray ionization (ESI) positive
- Curtain gas (CUR): 35 psi
- Nebulizer gas (GS1): 60 psi
- Voltage (IS): 5500 V
- Heater Temperature (TEM): 550 °C
- Turbo gas (GS2): 70 psi
- Collision gas (CAD): 8
- Dwell time: 50 ms

MRM mass transitions:

Azoxystrobin (quantifier) 404>372m/z

Azoxystrobin (qualifier) 404>329m/z

Prothioconazole (quantifier) 344>189m/z

Prothioconazole (qualifier) 344>154m/z

Qualifier was monitored but not evaluated.

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Azoxystrobin:

0.2 – 20 µg a.i./L (9 standard solutions)

The calibration range correspond to minus 71% of the lowest analysed concentration (LOQ) and plus 44% of the highest analysed concentration (nominal 12 mg test item/L).

$y = -84342 \cdot x^2 + 4770697 \cdot x + 1098113$ ($r = 0.9988$) - quantifier

$y = -101809 \cdot x^2 + 5021546 \cdot x + 703057$ ($r = 0.9993$) - qualifier

Prothioconazole:

0.24– 24 µg a.i./L (9 standard solutions)

The calibration range correspond to minus 72% of the lowest analysed concentration (LOQ) and plus 40% of the highest analysed concentration (nominal 12 mg test item/L).

$y = 36755 \cdot x + 2366$ ($r = 0.9998$) - quantifier

$y = -48 \cdot x^2 + 31555 \cdot x - 1269$ ($r = 1.0000$) - qualifier

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effect were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect (≥ 20 %) was observed.

Independently of the determined matrix effect, matrix-matched calibration standards were used for quantification.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) ≤ 20 % was obtained.

Azoxystrobin = 0.006 mg test item/L

Prothioconazole = 0.006 mg test item/L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 0.2 $\mu\text{g a.i./L}$

Prothioconazole = 0.24 $\mu\text{g a.i./L}$

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples and subsequent determination of the recoveries upon applying the test method. The recovery results obtained are provided in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Azoxystrobin	0.006 mg test item/L	97, 69, 75, 104, 91, 83, 73	88	17
Azoxystrobin	12 mg test item/L	112, 103, 102, 99, 106, 119, 105	107	6
Prothioconazole	0.006 mg test item/L	91, 88, 88, 92, 105, 69, 63, 71	83	17
Prothioconazole	12 mg test item/L	103, 106, 104, 101, 103, 124, 112, 112	108	7

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the test solutions fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the test solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQs for azoxystrobin and for prothioconazole were 0.01 mg test item/L.
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Reference: KCP 5.1.2/10

Report Azoxystrobin 160 g/L + Prothioconazole 200 g/L SC: Acute Toxicity to *Daphnia magna* in a Semi-Static 48-hour Immobilisation Test; Bauer, J. & Mollandin, G., 2024; Study No.: UPL/2024/0702

Guideline(s): Yes - SANTE/2020/12830 rev. 1
Deviations: No
GLP: Yes
Acceptability: Yes

Materials and methods

Samples were prepared by direct dilution of samples followed by analysis via LC-MS/MS.

Sample Preparation:

Biological treatment samples and control samples:

The samples were allowed to thaw to room temperature. They were then shaken well and treated with ultrasound for 1 minute to obtain homogenous samples. The samples were already diluted with D1 (acetonitrile/water/formic acid/cysteine hydrochloride, 400/600/2/0.2 (v/v/v/w)) by factor 1.25 directly after sampling. The samples were diluted further with D2 (Elendt "M4"/D1, 4/1, v/v) to match the calibration range, if necessary.

Fortified Samples:

Fortified samples were prepared on the day of analysis. Approximately 30 mg of the test item were homogeneously dispersed dissolved (5 minutes ultrasonication, 25 to 76 minutes stirring) in 50 mL test water to obtain a stock solution of approximately 0.6 g test item/L. Exact weighing and purity of the test item was considered for results evaluation. Three independent stock solutions were prepared. Appropriate amounts of these stock solutions were diluted with test water to obtain fortified samples at a level of 0.01 and 1.2 mg test item/L. Exact values were documented in the raw data.

The samples were shaken well and were diluted with D1 (acetonitrile/water/formic acid/cysteine hydrochloride, 400/600/2/0.2 (v/v/v/w)) by factor 1.25. The samples were diluted further with D2 (Elendt "M4"/D1, 4/1, v/v) to match the calibration range, if necessary.

LC-MS/MS Conditions

LC: Agilent Series 1290 pump and autosampler

Column: Phenomenex Kinetex C18 100 Å, 50 * 2.1 mm, 2.6 µm

Temperature: 40°C

Mobile Phase:

- A: HPLC water containing 0.12 % formic acid and 10 mM ammonium formate
- B: Methanol / HPLC water (95/5, v/v) containing 0.12 % formic acid and 10 mM ammonium formate

Gradient:

Time (min)	%A	%B
0.0	70	30
5.0	0	100
6.0	0	100
6.1	70	30
10	70	30

Run Time: 10 min

Flow Rate: 0.4 mL/min

Injection Volume: 20 µL

Detector: Mass spectrometer API 5500

Parameters:

- Ion Source: Electrospray ionization (ESI) positive
- Curtain gas (CUR): 35 psi
- Nebulizer gas (GS1): 60 psi
- Voltage (IS): 5500 V
- Heater Temperature (TEM): 550 °C

- Turbo gas (GS2): 70 psi
- Collision gas (CAD): 8
- Dwell time: 50 ms

MRM mass transitions:

Azoxystrobin (quantifier) 404>372m/z

Azoxystrobin (qualifier) 404>329m/z

Prothioconazole (quantifier) 344>189m/z

Prothioconazole (qualifier) 344>154m/z

Qualifier was monitored but not evaluated.

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Azoxystrobin:

0.3 – 12 µg a.i./L (8 standard solutions)

The calibration range correspond to minus 74% of the lowest analysed concentration (LOQ) and plus 38% of the highest analysed concentration (nominal 1.2 mg test item/L).

$y = -153812 \cdot x^2 + 5771193 \cdot x + 185134$ ($r = 0.9999$) - quantifier

$y = -123446 \cdot x^2 + 4872535 \cdot x - 94684$ ($r = 1.0000$) - qualifier

Prothioconazole:

0.36 – 14.4 µg a.i./L (8 standard solutions)

The calibration range correspond to minus 75% of the lowest analysed concentration (LOQ) and plus 34% of the highest analysed concentration (nominal 1.2 mg test item/L).

$y = 35448 \cdot x + 950$ ($r = 0.9998$) - quantifier

$y = 27900 \cdot x + 1081$ ($r = 0.9998$) - qualifier

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effects were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

Independently of the determined matrix effect, matrix-matched calibration standards were used for quantification.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation ($RSD \leq 20\%$) was obtained.

Azoxystrobin = 0.01 mg test item/L

Prothioconazole = 0.01 mg test item/L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 0.3 µg a.i./L

Prothioconazole = 0.36 µg a.i./L

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples and subsequent determination of the recoveries upon applying the test method. The recovery results obtained are provided in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Azoxystrobin	0.01 mg test item/L	75, 78, 82, 79, 84	80	4
Azoxystrobin	1.2 mg test item/L	113, 114, 117, 111, 112	113	2
Prothioconazole	0.01 mg test item/L	94, 102, 92, 123, 92	101	13
Prothioconazole	1.2 mg test item/L	114, 115, 116, 117, 114	115	1

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the test solutions fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the test solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQ was 13 g test item/L corresponding to 4.7 mg Azoxystrobin/L after dilution by factor 400 5.8 mg Prothioconazole/L after dilution by factor 400.
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Reference:	KCP 5.1.2/11
Report	Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects on Terrestrial (Non-Target) Plants: Vegetative Vigour Test; Stürtz, S. & Horstmann, C.; 2024; Study No.: UPL/2023/2350
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture (acetonitrile/pure water (50/50, v/v)) to match the calibration range. Samples were diluted while solutions were stirring, if necessary. The samples were analysed on the day of processing (therefore stability testing was not needed).

Fortification Procedure:

The test item was dissolved in pure water (stirred for 60 minutes) to get fortified samples of about 13 g test item/L and 26 g test item/L. Fortified samples were diluted with solvent mixture (acetonitrile/pure water (50/50, v/v)) to match the calibration range.

Samples were analysed using HPLC-UV-method (250 nm)

Chromatographic Conditions

HPLC-Conditions:

System:	Waters Acquity
Software:	Empower 3
Column:	Kinetex C18 (50 × 2.1 mm; 2.6 µm)
Mobile Phase:	A: acetonitrile + 0.1 % formic acid B: pure water + 0.1 % formic acid
Gradient Mode:	
	Time [min] 0-6
	Solvent A [%] 40
	Solvent B [%] 60
Flow Rate:	0.4 mL/min
Temperature:	25 °C

Injection Volume: 2 µL

Detector: DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm

Retention Time: Azoxystrobin: 2.7 minutes Prothioconazole: 4.1 minutes

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Calibration Range: 1 to 15 mg Azoxystrobin/L corresponding to 21 % of LOQ to 159 % of higher fortification level ($y = 12321x - 721$, $r = 1.0000$), and 1 to 15 mg Prothioconazole/L corresponding to 17 % of LOQ to 128 % of higher fortification level ($y = 10979x - 1854$, $r = 0.9999$). For each calibration plot, 7 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effects were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

Matrix matched standards were used for the determination of the active ingredients.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) $\leq 20\%$ was obtained.

Azoxystrobin = 13 g test item /L

Prothioconazole = 13 g test item /L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 1 mg/L

Prothioconazole = 1 mg/L

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples (pure water) and subsequent determination of the recoveries upon applying the test method. Acceptable recoveries were obtained, as shown in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Azoxystrobin	13 g test item/L	106, 107, 103, 104, 107	105	2
Azoxystrobin	26 g test item/L	105, 106, 104, 103, 105	105	1
Azoxystrobin	biological stock solution(21.38 g test item/L)	103	-	-
Prothioconazole	13 g test item/L	100, 100, 96, 97, 101	99	2
Prothioconazole	26 g test item/L	100, 100, 97, 97, 99	99	2
Prothioconazole	biological stock solution(21.38 g test item/L)	97	-	-

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the test solution fulfils the requirements of SANTE/2020/12830

and has shown to be valid.

Comments of zRMS:	The method was successfully validated for the determination of azoxystrobin and prothioconazole in the test solutions in accordance to guidance document SANTE/2020/12830, rev.2 for risk assessment. The LOQ was 13 g test item/L corresponding to 4.7 mg Azoxystrobin/L after dilution by factor 400, 5.8 mg Prothioconazole/L after dilution by factor 400.
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Reference:	KCP 5.1.2/12
Report	Azoxystrobin 160 + Prothioconazole 200 g/L SC: Effects on Terrestrial (Non-Target) Plants: Seedling Emergence and Seedling Growth Test; Stürtz, S. & Horstmann, C.; 2024; Study No.: UPL/2023/2349
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Sample Preparation:

After thawing, an aliquot of each sample was diluted with solvent mixture (acetonitrile/pure water (50/50, v/v)) to match the calibration range. Samples were diluted while solutions were stirring, if necessary. The samples were analysed on the day of processing (therefore stability testing was not needed).

Fortification Procedure:

The test item was dissolved in pure water (stirred for 60 minutes) to get fortified samples of about 13 g test item/L and 26 g test item/L. Fortified samples were diluted with solvent mixture (acetonitrile/pure water (50/50, v/v)) to match the calibration range.

Samples were analysed using HPLC-UV-method (250 nm)

Chromatographic Conditions

HPLC-Conditions:

System:	Waters Acquity
Software:	Empower 3
Column:	Kinetex C18 (50 × 2.1 mm; 2.6 µm)
Mobile Phase:	A: acetonitrile + 0.1 % formic acid B: pure water + 0.1 % formic acid
Gradient Mode:	
Time [min]	0-6
Solvent A [%]	40
Solvent B [%]	60
Flow Rate:	0.4 mL/min
Temperature:	25 °C
Injection Volume:	2 µL
Detector:	DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm
Retention Time:	Azoxystrobin: 2.7 minutes Prothioconazole: 4.1 minutes

Results and discussions

Specificity

No interference above 30% of LOQ was observed in control samples at retention times of target analytes.

Linearity

Calibration Range: 1 to 15 mg Azoxystrobin/L corresponding to 21 % of LOQ to 159 % of higher fortification level ($y = 12321x - 721$, $r = 1.0000$), and 1 to 15 mg Prothioconazole/L corresponding to 17

% of LOQ to 128 % of higher fortification level ($y = 10979x - 1854$, $r = 0.9999$). For each calibration plot, 7 calibration standards were used. Samples were diluted to fit within the calibration range.

The regression residuals are randomly distributed, and no trends are visible.

Matrix effects

Matrix effects were assessed by comparing the response of the analyte in form of peak area in matrix standard solutions to standard solutions prepared in solvent. No significant matrix effect ($\geq 20\%$) was observed.

Matrix matched standards were used for the determination of the active ingredients.

Limit of Quantification (LOQ)

The LOQ was determined as the lowest fortification level at which an acceptable mean recovery (70 to 120 % of nominal) with a relative standard deviation (RSD) $\leq 20\%$ was obtained.

Azoxystrobin = 13 g test item/L

Prothioconazole = 13 g test item /L

Limit of Detection (LOD):

The LOD is defined as the lowest calibration standard concentration.

Azoxystrobin = 1 mg/L

Prothioconazole = 1 mg/L

Accuracy and precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated matrix samples (pure water) and subsequent determination of the recoveries upon applying the test method. Acceptable recoveries were obtained, as shown in the following table:

Analyte	Fortification	Recovery (%)	Mean recovery (%)	%RSD
Azoxystrobin	13 g test item/L	106, 107, 103, 104, 107	105	2
Azoxystrobin	26 g test item/L	105, 106, 104, 103, 105	105	1
Azoxystrobin	biological stock solution(21.38 g test item/L)	103	-	-
Prothioconazole	13 g test item/L	100, 100, 96, 97, 101	99	2
Prothioconazole	26 g test item/L	100, 100, 97, 97, 99	99	2
Prothioconazole	biological stock solution(21.38 g test item/L)	97	-	-

No residues above the LOD were observed in the blank matrix samples.

Conclusion

The method for the analysis of FGF11 in the test solution fulfils the requirements of SANTE/2020/12830 and has shown to be valid.

Comments of zRMS:	The analytical method principle was based on 01062/M004 (the method used in the dRAR for the prothioconazole renewal). The method was successfully validated for determination of all analytes (TDMs) in honey with an LOQ of 0.01 mg/kg according to guidance document SANTE/2020/12830, rev. 2.
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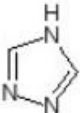
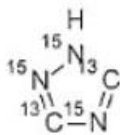
Reference: KCP 5.1.2/13

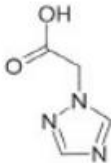
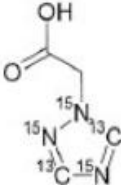
Report Magnitude of the residue of triazole derivative metabolites in phacelia honey in Northern and Southern Europe – 2023; Frédéric Lebrun; 2024;

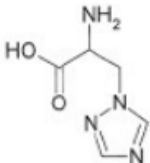
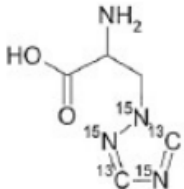
	Study No.: 644-2023
Guideline(s):	Yes - SANTE/2020/12830 rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

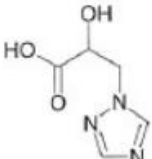
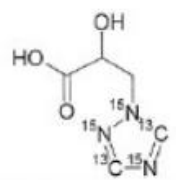
Materials and methods

Reference items

Reference Item 1			
Common name	1,2,4-Triazole	Synonym (s)	1,2,4-T; T
Chemical name (IUPAC)	1H-1,2,4-Triazole		
Chemical structure		CAS number	288-88-0
		Empirical formula	C ₂ H ₃ N ₃
		Molecular weight (average)	69.1 g/mol
Supplier	HPC Standards GmbH	Purity analysed	99.9 %
		Batch ID	791549
Date of certificate	01 Oct 2019	Expiry date	01 Oct 2024
Date of receipt at Test Facility	18 Dec 2019	Storage conditions at Test Facility	≤ -18 °C
Internal Standard			
Common name	1,2,4-Triazole ISTD	Synonym (s)	1,2,4-T ISTD, 1,2,4-T IL-IS
Chemical name (IUPAC)	4H-1,2,4-triazole- ¹³ C ₂ - ¹⁵ N ₃		
Chemical structure		Empirical formula	¹³ C ₂ H ₃ ¹⁵ N ₃
		Molecular weight (average)	74.0 g/mol
Supplier	Selcia Ltd.	Purity analysed	99.4 %
Date of certificate	27 Oct 2021	Batch number	11690AJT001-1
Date of receipt at Test Facility	06 Oct 2020	Storage conditions at Test Facility	≤ -18 °C

Reference Item 2			
Common name	Triazole acetic acid	Synonym (s)	TAA
Chemical name (IUPAC)	[1,2,4] Triazol-1-yl-acetic acid		
Chemical structure		CAS number	28711-29-7
		Empirical formula	C ₄ H ₅ N ₃ O ₂
		Molecular weight	127.1 g/mol
Supplier	HPC Standards GmbH	Purity analysed	98.9 %
Date of certificate	24 Jan 2020	Batch ID	793294
		Expiry date	01-Feb-2025
Date of receipt at Test Site	06-Oct-2020	Storage conditions at Test Site	≤ -18 °C
Internal Standard			
Common name	Triazole acetic acid ISTD	Synonym (s)	TAA ISTD, TAA IL-IS
Chemical name (IUPAC)	2-(1,2,4-[¹³ C ₂ , ¹⁵ N ₃]triazol-1-yl)acetic acid		
Chemical structure		Empirical formula	¹³ C ₂ H ₅ ¹⁵ N ₃ O ₂
		Molecular weight	132.1 g/mol
Supplier	Selcia Ltd	Purity analysed	98.1 %
Date of certificate	09-Nov-2021	Batch number	11692AJT001-1
Date of receipt at Test Site	06-Oct-2020	Storage conditions at Test Site	≤ -18 °C

Reference Item 3			
Common name	Triazole alanine	Synonym (s)	TA
Chemical name (IUPAC)	2-Amino-3-(1,2,4-Triazol-1-yl) propanoic acid		
Chemical structure		CAS number	114419-45-3
		Empirical formula	C ₅ H ₆ N ₄ O ₂
		Molecular weight	156.1 g/mol
Supplier	HPC Standards GmbH	Purity analysed	97.1 %
Date of certificate	30-May-2022	Batch ID	810264
		Expiry date	01-Jun-2027
Date of receipt at Test Facility	25-Oct-2022	Storage conditions at Test Facility	≤ -18 °C
Internal Standard			
Common name	Triazole alanine ISTD	Synonym (s)	TA ISTD, TA-IL-IS
Chemical name (IUPAC)	2-amino-3-(1,2,4-[¹³ C ₂ , ¹⁵ N ₃]-triazol-1-yl) propanoic acid		
Chemical structure		Empirical formula	¹³ C ₂ C ₃ H ₅ ¹⁵ N ₃ NO ₂
		Molecular weight	161.1 g/mol
Supplier	Selcia Ltd.	Purity analysed	99.6 %
Date of certificate	09-Nov-2021	Batch number	11691AJT001-1
Date of receipt at Test Facility	06-Oct-2020	Storage conditions at Test Facility	≤ -18 °C

Reference Item 4			
Common name	Triazole lactic acid hydrochloride	Synonym (s)	TLA HCl
Chemical name (IUPAC)	2-hydroxy-3-(1,2,4-triazol-1-yl)propanoic acid hydrochloride		
Chemical structure		CAS number	2126162-19-2
		Empirical formula	C ₅ H ₄ ClN ₃ O ₃
		Molecular weight	193.6 g/mol
Supplier	HPC Standards GmbH	Purity analysed	78.05 %
Date of certificate	02-Sep-2021	Batch ID	804947
		Expiry date	01 Sep 2026
Date of receipt at Test Site	28-Oct-2022	Storage conditions at Test Site	≤ -18°C
Internal Standard			
Common name	Triazole lactic acid ISTD	Synonym (s)	TLA ISTD, TLA-IL-IS
Chemical name (IUPAC)	2-hydroxy-3-[(¹³ C ₂ , ¹⁵ N ₃)-1 <i>H</i> -1,2,4-triazol-1-yl] propanoic acid		
Chemical structure		Empirical formula	¹³ C ₂ C ₃ H ₇ ¹⁵ N ₃ O ₃
		Molecular weight	162.1 g/mol
Supplier	Selcia Ltd.	Purity analysed	90.9 %
Date of certificate	09 Nov 2021	Batch number	11693AJT001-1
Date of receipt at Test Site	06-Oct-2020	Storage conditions at Test Site	≤ -18°C

I. Reagents and Materials

Information pertaining to the identity and source of reagents typically used is summarised in Table I. Unless specifically stated otherwise, equivalent reagents and materials may be used as long as the method's performance is not affected. Verifications were not performed as part of this analytical phase.

Table I. Identification of Reagents and Materials

- Formic acid
(Riedel de Haen, Art. No. 33015)
- Methanol
(Chromasolv, Sigma-Aldrich, Art. No. 34860)
- Water
(ultra-pure water, Purelab Flex 2, Elga Veolia)

II. Instrumentation and Apparatus

Information pertaining to the identity of instrumentation and apparatus typically used is summarised in Table II. Unless specifically stated otherwise, equivalent instrumentation and apparatus may be used as long as the method's performance is not affected. Verifications were not performed as part of this analytical phase.

Table II. Identification of Instrumentation and Apparatus

- | |
|---|
| <ul style="list-style-type: none">• Adjustable pipettes
(Reference 10-100 µL and Reference, 100-1000 µL, Eppendorf and HandyStep with 12.5 mL tip, Brand)• Bottles, wide neck, with screw cap, 150mL
(VWR, Art. No. 215-5632)• Common laboratory glassware• Cotton wool wadding
(Hartmann, Art. No. BODE1101292)• Funnels, analytical 65 mm
(VWR, Art. No. 221-0246)• Homogenizer
(Polytron PT 3100, KINEMATICA AG)• HPLC Autosampler vials, 1.8 mL• Nitrogen Evaporator
(N-EVAP, Organomation Associates, Inc., Art. No. 11155-RT)• Test tubes, with ground stoppers and graduation marks at 2.5, 5.0 and 10 mL• Ultrasonic bath
(Bandelin Sonorex RK 510 H , BANDELIN electronic GmbH & Co. KG, Art. No. 321)• Volumetric pipettes, e.g. 1, 2 and 5 mL• Volumetric flasks, e.g. 10, 20 and 25 mL• Water bath
(linear shaking water bath, GFL GmbH, Art. No. 1083) |
|---|

III. Preparation of Standard Solutions

Stock solutions of the analytes and isotopically labelled standards were prepared by dissolving a weight of the reference items with the aid of an ultrasonic bath. Each stock solution was allocated a unique reference number.

Solutions for fortification and calibration were obtained by (serial) dilution of the stock solutions.

All solutions were stored at typically 1 °C to 10 °C in a glass vial in the dark.

A summary of exemplary stock solutions and dilutions is presented in the following tables.

Table III. Exemplary Stock Solutions

Reference of solution produced	Analyte	Target amount of analyte corrected for purity (mg)	Solvent	Final volume (mL)	Target final concentration (µg/mL)
SL 17790	1,2,4-Triazole	10	Water/Methanol (4+1, v+v)	25	400
SL 17796	Triazole acetic acid	10	Water/Methanol (4+1, v+v)	25	400
SL 17792	Triazole alanine	4.0	Water/Methanol (4+1, v+v)	10	400
SL 17794	Triazole lactic acid hydrochloride	4.0	Water/Methanol (4+1, v+v)	10	400*
SL 17791	1,2,4-Triazole ISTD	10	Water/Methanol (4+1, v+v)	25	400
SL 17797	Triazole acetic acid ISTD	10	Water/Methanol (4+1, v+v)	25	400
SL 17793	Triazole alanine ISTD	10	Water/Methanol (4+1, v+v)	25	400
SL 17795	Triazole lactic acid ISTD	10	Water/Methanol (4+1, v+v)	25	400

* Expressed as triazole lactic acid = 325.2 µg/mL

Results and discussions

Table A 1: Recovery results from independent laboratory validation of TDMs using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Honey*	1,2,4-Triazole	0.01	99	10	
Honey**	1,2,4-Triazole	0.1	100	1.3	
Honey*	1,2,4-Triazole	0.01	97	6.0	
Honey**	1,2,4-Triazole	0.1	94	2.1	
Honey*	TAA	0.01	92	4.7	
Honey**	TAA	0.1	96	2.0	
Honey*	TAA	0.01	91	2.5	
Honey**	TAA	0.1	93	2.3	
Honey*	TA	0.01	93	3.4	
Honey**	TA	0.1	95	1.7	
Honey*	TA	0.01	96	4.7	
Honey**	TA	0.1	93	1.8	
Honey*	TLA	0.01	95	4.1	
Honey**	TLA	0.1	100	1.8	
Honey*	TLA	0.01	95	1.9	

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Honey**	TLA	0.1	94	1.3	

* hypercarb column

** aquasil C18 column

Table A 2: Characteristics for the analytical method used for independent laboratory validation of TDMs residues in honey

	1,2,4-Triazole	TAA	TA	TLA
Specificity	The blank values at the expected retention time of the analytes resulting from reagents and/or the control sample material used for recovery determinations did not exceed a level that would correspond to 30% of the LOQ.			
Calibration (type, number of data points)	8 calibrations points			
Calibration range	0.30 ng/mL to 30 ng/mL this range corresponds to 0.003 mg/kg to 0.3 mg/kg/			
Assessment of matrix effects is presented	Isotopically labelled internal standards were used for quantification so that possible matrix effects on the detector response are automatically compensated when using the response ratio of analyte to internal standard for quantification. Therefore, matrix effects on detection were not determined within this analytical phase.			
Limit of determination/quantification	0.01 mg/kg	0.01 mg/kg	0.01 mg/kg	0.01 mg/kg

Conclusion

The validation criteria for the method used to determine TDMs in honey has been met in accordance with SANTE/2020/12830 rev. 1.

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)

Independent laboratory validation

Comments of zRMS:	The primary method (J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)) was independently validated for the determination of prothioconazole-desthio in different matrices of plant origin (orange, cucumber, dried beans and oilseed rape seeds) according to the guidance document SANTE/2020/12830, rev. 1.							
	Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
	Mass Transition <i>m/z</i> 312→70 (Quantification)							
	Cucumber	0.005	94, 91, 94, 96, 92	93	2	5	96	3
		0.05	98, 99, 98, 98, 95	98	2	5		
	Mass Transition <i>m/z</i> 312→125 (Confirmation)							
	Cucumber	0.005	94, 92, 95, 95, 92	94	2	5	95	2
		0.05	97, 99, 97, 96, 95	97	2	5		
	Mass Transition <i>m/z</i> 312→70 (Quantification)							
	Orange	0.005	118, 115, 119, 111, 116	116	3	5	110	6
		0.05	101, 104, 105, 107, 103	104	2	5		
	Mass Transition <i>m/z</i> 312→125 (Confirmation)							
	Orange	0.005	119, 116, 118, 114, 116	117	2	5	110	6
		0.05	100, 105, 106, 107, 103	104	3	5		
	Mass Transition <i>m/z</i> 312→70 (Quantification)							
	Oilseed rape seeds	0.005	83, 82, 78, 84, 84	82	3	5	80	7
		0.05	73, 90, 76, 76, 76	78	9	5		
	Mass Transition <i>m/z</i> 312→125 (Confirmation)							
	Oilseed rape seeds	0.005	84, 83, 76, 84, 85	82	4	5	81	7
		0.05	71, 91, 78, 77, 76	79	9	5		
	Mass Transition <i>m/z</i> 312→70 (Quantification)							
	Dried beans	0.005	91, 85, 88, 89, 85	88	3	5	88	3
		0.05	88, 88, 84, 93, 90	89	4	5		
	Mass Transition <i>m/z</i> 312→125 (Confirmation)							
	Dried beans	0.005	92, 88, 91, 91, 90	90	2	5	90	3
		0.05	88, 90, 85, 93, 92	90	4	5		
	The limit of quantification was 0.005 mg/kg for prothioconazole-desthio in all plant matrices.							

Reference:	KCP 5.2/01
Report	Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Prothioconazole-desthio in Different Matrices of Plant Origin, N. Boubakri, 2023, Report No. S21-08354
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method used was in line with the primary method (J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)). There were no deviations.

Results and discussions

The method for the determination of prothioconazole-desthio in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 20: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Dried beans	Prothioconazole-desthio	0.005	88	3	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Dried beans	Prothioconazole-desthio	0.05	89	4	

Table A 21: Characteristics for the analytical method used for independent laboratory validation of prothioconazole residues in crops

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (> 30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points
Calibration range	0.150 – 6.0 ng/mL for dried beans equivalent to 0.0015 – 0.06 mg/kg
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD: 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion

The method for the determination of prothioconazole-desthio in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for the primary method (J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)).

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

Comments of zRMS:	The analytical method was successfully validated for the determination of prothioconazole-desthio (as sum of isomers) in five different matrices of food of animal origin (bovine whole milk, fat, liver, meat and poultry's eggs with LOQ of 0.004 mg/kg in milk and 0.01 mg/kg for the remaining matrices according to the guidance document SANTE/2020/12830, rev. 1 for monitoring.
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Reference: KCP 5.2/02

Report Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Food of Animal Origin, D. Kleinhenz, 2023, Report No. S21-08355

Guideline(s): Yes – SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of prothioconazole-desthio in food of animal origin was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Samples were extracted with acetonitrile after addition of water with a ratio of 2 mL of extraction solvent per g of matrix. Liquid/liquid partition was performed by addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation. Clean-up was performed by purification of an aliquot of the acetonitrile extract by dispersive SPE with primary/secondary amine (PSA) and freezing out for fat matrix. The sample concentration in final extracts was 0.5 g sample per mL of extract. Quantification was performed by LC-MS/MS.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu			
Pre-column	HPLC guard column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)			
Column	Kinetex® 5µm XB-C18 100 Å (150 mm x 4.6 mm, 5 µm, Phenomenex, Art. No. 00F-4605-E0)			
Column oven temperature	40 °C			
Injection volume	10 µL			
Mobile phases	Eluent A: Water + 0.1 % formic acid (v/v) Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0.0	40	60	1.0
	1.0	40	60	1.0
	5.0	10	90	1.0
	6.0	10	90	1.0
	6.1	0	100	1.0
	7.0	0	100	1.0
	7.1	40	60	1.0
	9.0	40	60	1.0
Divert valve	To mass spectrometer from 4.5 min to 6.5 min			
Retention time	Prothioconazole-desthio: approx. 5.5 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	5500 V	Ionspray turbo heater (TEM)		650 °C		
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)	Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)	Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
	Prothioconazole-desthio	312 → 70 [#] 312 → 125	126 126	10 10	47 49	12 6

[#] proposed for quantification. Both of the mass transitions listed can be used for quantification

Results and discussions

The analytical method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 312→70 and m/z 312→125). Due to enhanced sensitivity mass transition m/z 312→70 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.06 ng/mL to 6 ng/mL for milk and 0.15 ng/mL to 15 ng/mL for the remaining matrices. This range corresponds to a mass fraction level of 0.0012 mg/kg to 0.12 mg/kg for milk and 0.003 mg/kg to 0.3 mg/kg for the remaining matrices and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.999 .

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.004 mg/kg and 0.04 mg/kg for milk and 0.01 mg/kg to 0.1 mg/kg for the remaining matrices for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression or enhancement was $< \pm 20$ % for all matrices and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.004 mg/kg was confirmed for prothioconazole-desthio in milk and an LOQ of 0.01 mg/kg was confirmed for the remaining matrices. The LOD was defined as the lowest calibration standard, corresponding to 0.0012 mg/kg for milk and 0.003 mg/kg for the remaining matrices, which is 30 % of the LOQ.

Table A 22: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
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Milk	0.004	104	2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.04	97	2	
Egg	0.01	100	1	
	0.1	98	1	
Fat	0.01	85	4	
	0.1	83	3	
Liver	0.01	98	2	
	0.1	91	2	
Meat	0.01	101	2	
	0.1	97	2	

Table A 23: Characteristics for the analytical method used for validation of prothioconazole-desthio in food of animal origin

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 5 calibration points.
Calibration range	0.06 – 6 ng/ml for milk and 0.15 ng/ml – 15 ng/ml for the other matrices. This corresponds to 0.0012 – 0.12 mg/kg for milk and 0.003 – 0.3 mg/kg for the other matrices. $r \geq 0.999$
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	LOQ = 0.004 mg/kg (milk) and 0.01 mg/kg (all other matrices) LOD = 0.0012 mg/kg (milk) and 0.003 mg/kg (all other matrices)

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio in food of animal origin has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The primary method (D. Kleinhenz, 2023; Report No. S21-08355) was independently validated for the determination of prothioconazole-desthio in different matrices of animal origin (milk and fat) according to the guidance document SANTE/2020/12830, rev. 1. LOQ was 0.04 mg/kg for milk) and 0.01 mg/kg for fat.
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Reference:	KCP 5.2/03
Report	Independent Laboratory Validation of Prothioconazole-desthio in Different Matrices of Animal Origin, T. Rastogi, 2023, Report No. S21-08868
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method used was in line with the primary method above. There were no deviations.

Results and discussions

The method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 24: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Milk	0.004	81.0	11	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.04	88.2	6.8	
Fat	0.01	78.5	4.6	
	0.10	75.4	4.7	

Table A 25: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in food of animal origin

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points.
Calibration range	0.060 - 6.0 ng/mL for milk and 0.150 - 15.0 ng/mL for fat matrix. Corresponding to 0.0012 - 0.12 mg/kg for milk and 0.003 - 0.3 mg/kg for fat. $r \geq 0.99$
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.04 mg/kg (milk) and 0.01 mg/kg (fat) LOD = 0.0012 mg/kg (milk) and 0.003 mg/kg (fat)

Conclusion

The method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for D. Kleinhenz, 2023 (Report No. S21-08355).

Comments of zRMS:	The analytical methods were successfully validated for the determination of prothioconazole-desthio and five hydroxy metabolites in honey with LOQ of 0.005 mg/kg for each analyte according to the guidance document SANTE/2020/12830, rev. 1.
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Reference:	KCP 5.2/04
Report	Prothioconazole-desthio and hydroxy metabolites: Method Validation in Honey, J. Hitchens, 2023, Report No. QG/21/009
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Quantitative determination of prothioconazole-desthio and hydroxy metabolites in honey was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of prothioconazole-desthio were extracted from honey by dissolving the samples in water and then extracting the analyte with acetonitrile using QuEChERS kits. A portion of the final extract was then taken through the SPE clean-up and then diluted with water 2:3 v/v for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Residues of the prothioconazole-desthio (5) hydroxy metabolites were extracted from honey by dissolving the samples in water and then extracting the analyte with acetonitrile using QuEChERS kits. The samples were then diluted with water, evaporated to the aqueous remainder through rotary evaporation followed by a hydrolysis step consisting of an overnight incubation at 40°C under acidic conditions. A portion of the final extract was then taken through liquid-liquid extraction, evaporated to dryness using nitrogen flow, reconstituted in acetonitrile, sonicated, and then diluted with water for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Example Instrument Conditions

Liquid Chromatography:

Column:	Kinetex 5 µm XB-C18 100A, 150 x 4.6 mm		
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287		
Column Oven Temperature (°C):	40		
Injection Volume * (µL):	60		
Flow Rate (µL/min):	1000		
Mobile Phase A:	0.1 % Formic acid in water		
Mobile Phase B:	0.1 % Formic acid in methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	40	60
	1.00	40	60
	5.00	10	90
	6.00	10	90
	6.10	0	100
	7.00	0	100
	7.10	40	60
	9.00	40	60
Flow divert:	Time (minutes)	Position	
	0.0	To waste	
	4.0	To mass spec	
	6.0 - End	To waste	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)		
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)		

Mass Spectrometry:

MS/MS System	API 5000
Ionisation Mode:	ESI
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	5500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10
Declustering Potential (DP)** (V)	126

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass*	Q3 Mass*	Dwell Time (msec)	Collision Energy* (CE) (V)	Collision Exit Potential* (CXP) (V)	Declustering Potential (DP)	Expected Retention time (±0.5mins)
Prothioconazole -desthio	1#	312.1	70.1	250	20	10	126	5.3
	2#	312.1	125.0	250	30	15	126	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

Results and discussions

The analytical method for the determination of prothioconazole-desthio and hydroxy metabolites in honey has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole-desthio and (5) hydroxy metabolites were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating good selectivity of the method. In addition, LC-MS/MS monitoring two ion mass transitions is a highly specific technique. Product ion spectra for all analytes were obtained to justify the selection of ions used for analytical determinations.

Linearity

Calibration curves were obtained from a minimum of 8 calibration solutions containing prothioconazole-desthio and (5) hydroxy metabolites. For prothioconazole-desthio the standard calibration range was 0.05 to 2.5 ng/mL (equivalent to 0.00125 – 0.0625 mg/kg), covering a range from 25% of the LOQ to 25% above the highest concentration level.

For prothioconazole-desthio hydroxy metabolites the standard calibration range was 0.375 to 15.0 ng/mL (equivalent to 0.0015 – 0.060 mg/kg), covering a range from 30% of the LOQ to 20% above the highest concentration level.

Matrix matched standards were prepared with the exception of the extract stability analytical batch. Correlation coefficients $r \geq 0.995$ and coefficients of determination (R^2) ≥ 0.99 were achieved in the validation and extract stability batches using a linear regression with a 1/x weighting, demonstrating acceptable linearity.

Accuracy and Precision

Average recoveries and the overall average recovery were all within the acceptance range of 60-120% at the LOQ fortification level and 70-120% at the 10xLOQ fortification level, demonstrating satisfactory accuracy. The relative standard deviation (RSD) and the overall RSD did not exceed 30% at the LOQ fortification level and did not exceed 20% at the 10xLOQ fortification level, demonstrating satisfactory precision.

Matrix Effects

Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). No significant matrix effects were observed. (i.e. >20% enhancement or suppression). However, matrix matched standards were used with the exception of the extract stability analytical batch.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

Prothioconazole-desthio: the limit of detection (LOD) was 0.00125 mg/kg (25% of LOQ) equivalent to the lowest calibration standard. Prothioconazole-desthio (5) hydroxy metabolites: the limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole-desthio and (5) hydroxy metabolites in honey, as confirmed by recovery efficiency testing.

Table A 26: Characteristics for the analytical method used for validation of prothioconazole-desthio and hydroxy metabolites in honey

	Prothioconazole-desthio	Prothioconazole-desthio hydroxy metabolites
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio and (5) hydroxy metabolites were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples,	
Calibration (type, number of data points)	Minimum of 8 calibration points	
Calibration range	0.05 – 2.5 ng/ml (equivalent to 0.00125 – 0.0625 mg/kg) r ≥ 0.99	0.375 – 15.0 ng/ml (equivalent to 0.0015 – 0.060 mg/kg) r ≥ 0.99
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOD = 0.00125 mg/kg LOQ = 0.005 mg/kg	LOD = 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio and hydroxy metabolites in honey has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The primary method (J. Hitchens, 2023, Report No. QG/21/009) was independently validated for the determination of prothioconazole-desthio in honey with LOQ of 0.005 mg/kg according to the guidance document SANTE/2020/12830, rev. 1.
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Reference: KCP 5.2/05

Report Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Honey, J. Wanger, 2023, Report No. S21-08357

Guideline(s): Yes – SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The method used was in line with the primary method. There were no deviations.

Results and discussions

The method for the determination of prothioconazole-desthio in honey has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 27: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Honey	0.005	105	2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.05	110	2	

Table A 28: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in honey

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 9 calibration points.
Calibration range	0.050 - 3.0 ng/mL, corresponding to 0.00125 - 0.075 mg/kg r ≥ 0.999
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.005 mg/kg LOD = 0.00125 mg/kg

Conclusion

The method for the determination of prothioconazole-desthio in honey has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for J. Hitchens, 2024, Report No. QG/21/008 and J. Hitchens, 2023, Report No. QG/21/009.

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

Comments of zRMS:	The analytical method was successfully validated for the determination of prothioconazole and prothioconazole-desthio in soil with LOQ of 0.006 mg/kg for prothioconazole and 0.002 mg/kg for prothioconazole-desthio according to the guidance document SANTE/2020/12830, rev. 1. All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %. The method is acceptable.
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Reference: KCP 5.2/06

Report: Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Soil, M. Kaiser, 2022, Report No. S21-08358

Guideline(s): Yes – SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of prothioconazole and prothioconazole-desthio in soil was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	1290 Infinity II Binary LC System, Agilent Technologies			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)			
Column	Agilent ZORBAX Eclipse XDB-C18 (50 x 4.6 mm, 1.8 µm, Part No. 927975-902)			
Column oven temperature	40 °C			
Injection volume	40 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	80	20	400
	0.3	80	20	400
	4.0	10	90	400
	6.5	10	90	400
	6.51	80	20	400
	8.5	80	20	400
Divert valve	to mass spectrometer from 5.5 min to 8.2 min			
Retention times	Prothioconazole: approx. 6.5 min Prothioconazole-desthio: approx. 6.2 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX API 6500+ System, AB SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V	Ionspray turbo heater (TEM)		450 °C		
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)	Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)	Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 189 [#]	66	10	29	22	50
	344 → 154	66	10	43	16	50
Prothioconazole-desthio	312 → 70 [#]	101	10	59	10	50
	312 → 125	101	10	45	14	50

[#] proposed for quantification. Both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole and prothioconazole-desthio in soil has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 344→189 and m/z 344→154 for prothioconazole and m/z 312→70 and m/z 312→125 for prothioconazole-desthio). For prothioconazole mass transition m/z 344→189 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation and for prothioconazole-desthio m/z 312→70 accordingly.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to > 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.036 ng/mL to 36 ng/mL for prothioconazole and 0.12 ng/mL to 12 ng/mL for prothioconazole-dsestio. This range corresponds to a mass fraction level of 0.0018 mg/kg to 0.18 mg/kg and 0.006 mg/kg to 0.6 mg/kg respectively and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99 .

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.002, 0.006, 0.02 and 0.06 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression or enhancement was $> \pm 20$ % for all matrices and thus deemed to be significant. Therefore, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ in soil of 0.006 mg/kg was confirmed for prothioconazole and an LOQ of 0.002 mg/kg for prothioconazole-dsestio. The LOD was defined as the lowest calibration standard, corresponding to 0.002 mg/kg for prothioconazole and 0.0006 mg/kg for prothioconazole-dsestio, which is 30 % of the LOQ.

Table A 29: Recovery results from method validation of prothioconazole and prothioconazole-dsestio using the analytical method

Desthio using the analytical method				
Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Soil	0.006	81	9	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.06	92	3	
Prothioconazole-desthio				

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Soil	0.002	104	12	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.02	109	4	

Table A 30: Characteristics for the analytical method used for validation of prothioconazole and prothioconazole-desthio in soil

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.36 – 36 ng/mL, equivalent to 0.0018 - 0.18 mg/kg $r \geq 0.99$	0.12 - 12 ng/mL, equivalent to 0.006 - 0.6 mg/kg $r \geq 0.99$
Assessment of matrix effects is presented	Yes	
Limit of determination/quantification	LOQ = 0.006 mg/kg LOD = 0.002 mg/kg	LOQ = 0.002 mg/kg LOD = 0.0006 mg/kg

Conclusion

The validation criteria for the method used to determine prothioconazole and prothioconazole-desthio in soil has been met in accordance with SANTE/2020/12830 rev. 1.

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

Comments of zRMS:	The analytical method was successfully validated for the determination of prothioconazole and prothioconazole-desthio in drinking water and surface water with LOQ of 0.05 µg/L for both analytes according to the guidance document SANTE/2020/12830, rev. 1. All mean recoveries were within the acceptable range of 70 – 120% with RSD ≤ 20%. The method is acceptable.
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Reference: KCP 5.2/07

Report Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Water, M. Kaiser, 2022, Report No. S21-08359

Guideline(s): Yes – SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of prothioconazole and prothioconazole-desthio in drinking water and surface water was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	a) HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu b) 1290 Infinity II HPLC, Agilent			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)			
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl (50 x 2.1 mm, 1.8 µm, Part No. 959757-912)			
Column oven temperature	40 °C			
Injection volume	40 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	60	40	400
	0.3	60	40	400
	1.3	20	80	400
	4	5	95	400
	6.5	5	95	400
	6.51	60	40	400
	8.5	60	40	400
Divert valve	a) to mass spectrometer from 1.7 min to 3.0 min b) to mass spectrometer from 1.0 min to 3.5 min			
Retention times	Prothioconazole: approx. 2.4 min Prothioconazole-desthio: approx. 2.2 min			

Mass Spectrometry:

Mass spectrometric conditions (both matrices)						
MS system	a) SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer) b) SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive Ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)	Ionspray turbo heater (TEM)		450 °C		
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)	Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)	Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 154 [#]	66	10	43	16	50
	344 → 189	66	10	29	22	50
Prothioconazole-desthio	312 → 70 [#]	101	10	59	10	50
	312 → 125	101	10	45	14	50

[#] used for quantification, but both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole and prothioconazole-desthio in surface and drinking water has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions per analyte (m/z 344→189 and m/z 344→154 for prothioconazole and m/z 312→70 and m/z 312→125 for prothioconazole-desthio). For prothioconazole mass transition m/z 344→154 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation and for prothioconazole-desthio m/z 312→70 accordingly.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond

to > 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.0135 ng/mL to 1.35 ng/mL. This range corresponds to a mass fraction level of 0.0149 µg/kg to 1.49 µg/kg mg/kg respectively and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99 .

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.002, 0.006, 0.02 and 0.06 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Regarding prothioconazole matrix enhancement was at least partially $\geq + 20$ % and deemed to be significant for both matrices. Therefore, matrix matched standards were used for quantification throughout the study. Regarding prothioconazole-desthio, matrix effects were < 20 % for both matrices and this deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study for both matrices.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.05 µg/L was confirmed for prothioconazole and prothioconazole-desthio in drinking and surface water.

The LOD was defined as the lowest calibration standard, corresponding to 0.0149 µg/L for all matrices, which is ≤ 30 % of the LOQ.

Table A 31: Recovery results from method validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level ($\frac{\text{mg}}{\text{kg}}$ $\frac{\mu\text{g}}{\text{L}}$) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Drinking water	0.05	78	6	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	98	1	
Surface water	0.05	78	4	
	0.5	99	2	

Prothioconazole-desthio				
Drinking water	0.05	102	4	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	103	3	
Surface water	0.05	108	3	
	0.5	103	1	

Table A 32: Characteristics for the analytical method used for validation of prothioconazole and prothioconazole desthio residues in surface and drinking water

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.0135 – 1.35 ng/mL, equivalent to 0.0149 – 1.49 µg/L r ≥ 0.99	
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOQ = 0.05 µg/L LOD = 0.0149 µg/L	

Conclusion

The validation criteria for the method used to determine prothioconazole and prothioconazole-desthio in surface water and drinking water has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The primary method (M. Kaiser, 2022, Report No. S21-08359) was independently validated for the determination of prothioconazole and prothioconazole-desthio in drinking water with LOQ of 0.05 µg/L for both analytes according to the guidance document SANTE/2020/12830, rev. 1 for monitoring.
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Reference:	KCP 5.2/08
Report	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Water, S. Jooß, 2023, Report No. S21-08869
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method used was in line with the primary method. There were no deviations.

Results and discussions

The method for the determination of prothioconazole and prothioconazole-desthio in water has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 33: Recovery results from independent laboratory validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg µg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
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Prothioconazole				
Drinking water	0.05	97.5	4.2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	98	1	
Prothioconazole-desthio				
Drinking water	0.05	100	3	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	100	2.5	

Table A 34: Characteristics for the analytical method used for independent laboratory validation of prothioconazole and prothioconazole-desthio residues in water

	Prothioconazole and Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 6 calibration points.
Calibration range	0.0135 - 1.35 ng/mL, corresponding to 0.0149 – 2.49 µg/kg $r \geq 0.999$
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.05 µg/L LOD = 0.0149 µg/L

Conclusion

The method for the determination of prothioconazole and prothioconazole-desthio in water has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for M. Kaiser, 2023, Report No. S21-08359

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 analytical method was successfully validated for the determination of prothioconazole-desthio (as sum of isomers) in urine with LOQ of 0.01 mg/L according to the guidance document SANTE/2020/12830, rev. 1. All mean recoveries were within the acceptable range of 70 – 120% with RSD ≤ 20%. The method is acceptable.
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Reference: KCP 5.2/09

Report Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Urine, N. Boubakri, 2023, Report No, S21-08361

Guideline(s): Yes – SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Quantitative determination of prothioconazole-desthio in urine was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)			
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl (50 x 2.1 mm, 1.8 µm, Part No. 959757-912)			
Column oven temperature	40 °C			
Injection volume	5 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	60	40	400
	0.3	60	40	400
	1.3	20	80	400
	4	5	95	400
	6.5	5	95	400
	6.51	60	40	400
	8.5	60	40	400
Divert valve	to mass spectrometer from 1.7 min to 3.5 min			
Retention times	Prothioconazole-desthio: approx. 2.5 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive Ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)		Ionspray turbo heater (TEM)		450 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored	Decustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
	(m/z)					
Prothioconazole-desthio	312 → 70 [#]	101	10	59	10	50
	312 → 125	101	10	45	14	50

[#] proposed for quantification. Both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole-desthio in urine has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation by two (2) mass transitions (m/z 312→125 and m/z 312→70). Due to enhanced sensitivity mass transition m/z 312→70 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two (2) control samples were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference above 30 % of LOQ at the retention time of prothioconazole-desthio in urine, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.3 ng/mL to 30 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/L to 0.3 mg/L and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, correlation coefficients (R) were ≥ 0.99 .

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.01 mg/L and 0.1 mg/L for two (2) mass transitions are within 70 % - 120 % with relative standard deviations ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression was < 20 % for prothioconazole-desthio in urine and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/L was confirmed for prothioconazole-desthio in urine. The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/L, which is 30 % of the LOQ.

Table A 35: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (~~mg/kg~~ mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Urine	0.01	88	1	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.1	92	2	

Table A 36: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in urine

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification ($>30\%$ LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.03 ~~0.3~~ ng/mL – 30 ng/mL, equivalent to 0.003 mg/L – 0.3 mg/L $r \geq 0.99$	
Assessment of matrix effects is presented	yes	

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Limit of determination/quantification	LOQ = 0.01 mg/L LOD = 0.003 mg/L	

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio in urine has been met in accordance with SANTE/2020/12830 rev. 1.

Other Studies/ Information

No new or additional studies have been submitted.