

REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: GPF515/ FGE03

Product name(s): Boscalid / Prothioconazole 350 / 200 WG

Chemical active substance(s):

Boscalid, 350 g/kg

Prothioconazole, 200 g/kg

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(Article 33 application for a new product registration)

Applicant: UPL Holdings Coöperatief U.A.

Submission date: November 2024

MS Finalisation date: June 2025, updated October 2025 (initial Core Assessment)

November 2025 (final Core Assessment)

Version history

When	What
November 2024	V0 – Version from applicant for submission to z-RMS Poland in the frame of the PPP Authorization according to Article 33 of Regulation (EC) No 1107/2009.
June 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.
August 2025	V1 – Version from applicant for submission to z-RMS Poland in the frame of the PPP Authorization according to Article 33 of Regulation (EC) No 1107/2009. Update included after commenting period are highlighted in green .
September 2025	V2 – Version from applicant for submission to z-RMS Poland in the frame of the PPP Authorization according to Article 33 of Regulation (EC) No 1107/2009. Update included after commenting period are highlighted in yellow .
September 2025	V3 – Version from applicant for submission to z-RMS Poland in the frame of the PPP Authorization according to Article 33 of Regulation (EC) No 1107/2009. Update included after commenting period are highlighted in blue .
October 2025	Core Assessment updated following the commenting period and adding an assessment of new studies Additional information/assessments included by the zRMS in the report in response to comments received from the cMS and the Applicant are highlighted in yellow . 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.
November 2025	Final report (Core Assessment updated following the commenting period) Additional information/assessments included by the zRMS in the report in response to comments received from the cMS and the Applicant are highlighted in green . Not agreed or not relevant information are ~~struck through~~ and ~~shaded~~ for transparency.

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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 substance(s) and relevant impurities in the plant protection product.

Noticed data gaps are: none

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

Noticed data gaps are:

- The lack of an analytical method for monitoring purposes for determination of boscalid in hops, spices and herbal infusions has been identified during review of the Maximum Residue Levels (EFSA Journal 2014;12(7):3799)

~~This is post registration requirement.~~

This data gap should be addressed during the renewal of boscalid.

Commodity / crop	Supported / Not supported
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 boscalid, and prothioconazole in plant protection product is provided. The following studies have not been evaluated previously to Uniformed Principles and have been provided in support of this assessment.

Comments of zRMS:	Study acceptable. The analytical method for the determination of boscalid, 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 ingredient content of boscalid 350 g/kg + prothioconazole 200 g/kg WG, Desai H., 2023, Report No. 228-2-12-33016, UPL/2023/0300
Guideline(s):	Yes, SANCO/3030/99 rev.5 (March 22, 2019)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The active ingredients content of Boscalid 350 g/Kg + Prothioconazole 200 g/Kg WG are determined by high performance liquid chromatography on a reversed phase column Inertsil, ODS-3 using Acetonitrile (55%): 0.1% Ortho-phosphoric acid in water (45%), v/v, as mobile phase and UV detection at 220 nm.

The test item solution is prepared in 1mL Milo-Q water + Acetonitrile, sonicated for 5 minutes and filtered through 0.22 µm filter. The reference standard solutions were prepared by dissolving the corresponding quantity in acetonitrile.

Instrumental Parameters:

Instrument	HPLC (Shimadzu, LC-2010 CHT With Lab Solution Software)
Column	Inertsil, ODS-3 [150 mm x 4.6 mm (i.d), 5 µ particle size]
Wavelength	220 nm
Mobile Phase	Acetonitrile (55%): 0.1% Ortho-phosphoric acid in water (45%), v/v
Injection Volume	5.0 µL
Column Temperature	40 °C
Flow Rate	1.0 mL/minute

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances boscalid and prothioconazole in plant protection product GPF515 / FGE03

		Boscalid			Prothioconazole			
Author(s), year		Desai H., 2023						
Principle of method		HPLC-UV						
Parameters		Results						
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)		250.51 to 449.43 mg/L (24.75 % to 44.4%) (corresponding to 71- 127% of the nominal concentration)				102.6 to 300.10 mg/L (10.14 % to 29.65 %) (corresponding to 50- 148% of the nominal concentration)		
		For Repeatability-1 y= 19271.64 x + 230118.83 r= 0.999	For Repeatability-2 and Accuracy y= 19428.30 x + 182848.67 r= 0.999		For Repeatability-1 y= 10130.54 x + 17248.40 r= 0.999	For Repeatability-2 and Accuracy y= 10089.53 x + 7640.53 r= 0.999		
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability-1	Mean content (%w/w): 35.93 % RSD = 0.19 (n=5) Horwitz RSDr = 1.56 Horrat value (Hr) = 0.12				Mean content (%w/w): 19.95 % RSD = 0.50 (n=5) Horwitz RSDr = 1.71 Horrat value (Hr) = 0.29		
	Repeatability-2	Mean content (%w/w): 35.83 % RSD = 0.11 (n=5) Horwitz RSDr = 1.56 Horrat value (Hr) = 0.07				Mean content (%w/w): 20.03 % RSD = 0.15 (n=5) Horwitz RSDr = 1.71 Horrat value (Hr) = 0.09		
	Intermediate Precision	Mean content (%w/w): 35.88 % RSD = 0.20 (n=10) Horwitz RSDr = 2.33				Mean content (%w/w): 19.99 % RSD = 0.40 (n=10) Horwitz RSDr = 2.55		
Accuracy n = 9 (% Recovery)		Level (mg/L) / Mean % Recovery				Level (mg/L) / Mean % Recovery		
		I (288.06)	II (361.42)	III (431.59)		I (162.24)	II (207.59)	III (241.76)
		99.72	101.38	101.57		101.36	101.72	99.78
		Overall Mean % Recovery: 100.89				Overall Mean % Recovery: 100.95		
Interference/ Specificity		No interference. Chromatograms of blank formulation, standard solution, test item and fortified solutions are included in study report.						
Comment		The analytical method for the determination of Boscalid and Prothioconazole in the plant protection product GPF515 / FGE03 was accurately validated covering specificity, linearity, precision and recovery (accuracy) according with guideline SANCO/3030/99 rev.5.						

Conclusion

The analytical method provides a specific determination of the active ingredients boscalid and

prothioconazole in the formulation GPF515 / FGE03 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:

The following studies have not been evaluated previously to Uniformed Principles and have been provided in support of this assessment.

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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Relevant impurity: Toluene and Prothioconazole-Desthio

Reference:	KCP 5.1.2/01
Report	Validation of Analytical Method for Determination of Associated Impurities of Boscalid 350 g/kg + Prothioconazole 200 g/kg WG, Desai, H., 2024, Report No. 228-2-12-37860, UPL/2024/1238
Guideline(s):	Yes, SANCO/3030/99 rev.5 (March 22, 2019)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The associated impurities (Prothioconazole-Desthio and Toluene) of Boscalid 350 g/Kg + Prothioconazole 200 g/Kg WG were determined by high performance liquid chromatography on a reversed phase column Sunniest, C-18 using Acetonitrile: 0.1% Orthophosphoric acid (OPA) in Milli Q water (Gradient), as mobile phase and UV detection at 210 nm and GC-MS with VF-624 MS column using Helium at a flow rate of 1.0 mL/min.

Test item and reference standard solution preparation:

For HPLC:

The test item solution is prepared in Acetonitrile (507.12 mg test item dissolved in 10 mL), sonicated for 5 minutes and filtered through 0.22 µm filter. The reference standard solutions were prepared by dissolving the corresponding quantity in acetonitrile.

For GC-MS:

The test item solution is prepared in Acetonitrile (501.41 mg test item dissolved in 10 mL), sonicated for 5 minutes and filtered through 0.22 µm filter. The reference standard solutions were prepared by dissolving the corresponding quantity in acetonitrile.

Instrumental Parameters:

HPLC-MS/MS

Instrument	LC-MS-MS (API 4000 Mass Spectrometer coupled with Nexera X2 HPLC System, Analyst software version 1.6.3)
Column	Sunniest, C-18, (250 mm x 4.6 mm (i.d.), 5 µm particle size)
Column Temperature	35 °C
Wavelength	210 nm
Mobile Phase	Acetonitrile (55%): 0.1% Ortho-phosphoric acid in water (45%), v/v
Injection Volume	10 µL
Flow Rate	1.4 mL/minute
Mobile Phase	Acetonitrile: 0.1% Formic acid in Milli-Q water (Gradient)

GC-MS

Instrument	GC-MS (Agilent Technologies, 7890B/5977A MSD, Software: Maas Hunter, Software version B.07.05.2479)
Column	VF-624 MS [30 m x 0.25 mm (i.d.) x 0.25 µm film thickness]
Flow Rate	1.0 mL/minute
Carrier gas	Helium
Injection Volume	1.0 µL
Split Ratio	1:10
Temperature	
Column	40 °C (hold time 0 minutes) to 120 °C @ 20 °C/minute (hold time 2.0 minutes) to 240 °C @ 30 °C/minute (hold time 15.0 minutes)
Auxiliary Temperature	280 °C

Mass parameters

Analysis Mode Type	Scan
Mass Range	50 to 550 m/z
Quadrupole temperature	150 °C
MS Source Temperature	230 °C

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) GPF515 / FGE03

	Prothioconazole-Desthio max. content in PPP: 0.01% w/w		Toluene max. content in PPP: 0.1% w/w	
Author(s), year	Desai, H., 2024		Desai, H., 2024	
Principle of method	LC-MS/MS		GC-MS	
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	For Repeatability-1 Concentration Range (mg/L): 0.32 to 19.85 Intercept with y-axis (a): -866.90 Slope of the Line (b): 18084.63 Correlation Coefficient: 0.999	For Repeatability-2 and % Recovery Concentration Range (mg/L) : 0.32 to 19.85 Intercept with y-axis (a): 1899.03 Slope of the Line (b) 17381.45 Correlation Coefficient: 0.999	For Repeatability-1 Concentration Range (mg/L): 0.24 to 20.06 Intercept with y-axis (a): -1427.17 Slope of the Line (b): 28862.00 Correlation Coefficient: 0.999	For Repeatability-2 and % Recovery Concentration Range (mg/L) : 00.24 to 20.06 Intercept with y-axis (a): -2432.35 Slope of the Line (b) 28679.12 Correlation Coefficient: 0.999
	Concentration (%w/w): 0.00062 to 0.03856		Concentration (%w/w): 0.00047 to 0.03897	
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability-1	Mean Content ± SD: 0.00422 ± 0.00010% w/w % RSD: 2.37 Acceptable % RSD (Extended Horwitz): 6.10 Horrat Value (Hr): 0.39 Acceptable Horrat Value (Hr): ≤ 1	Repeatability-1	Mean Content ± SD: 0.01318 ± 0.00036% w/w % RSD: 2.73 Acceptable % RSD (Extended Horwitz): 5.14 Horrat Value (Hr): 0.53 Acceptable Horrat Value (Hr): ≤ 1
	Repeatability-2	Mean Content ± SD: 0.00415 ± 0.00017% w/w % RSD: 4.10 Acceptable % RSD (Extended Horwitz): 6.12 Horrat Value (Hr): 0.67 Acceptable Horrat Value (Hr): ≤ 1	Repeatability-2	Mean Content ± SD: 0.01343 ± 0.00035% w/w % RSD: 2.61 Acceptable % RSD (Extended Horwitz): 5.13 Horrat Value (Hr): 0.51 Acceptable Horrat Value (Hr): ≤ 1
	Intermediate Precision	Overall Mean Content ± SD: 0.00419 ± 0.00014% w/w (% RSD): 3.34 Acceptable % RSD (Horwitz): 9.12	Intermediate Precision	Overall Mean Content ± SD: 0.01330 ± 0.00036% w/w (% RSD): 2.71 Acceptable % RSD (Horwitz): 7.66

	Prothioconazole-Desthio max. content in PPP: 0.01% w/w			Toluene max. content in PPP: 0.1% w/w		
Accuracy n = 5 (% Recovery)	Level (mg/L)	(I -LOQ) (0.00078%)	(II- ≥10LOQ) (0.00777%)	Level (mg/L)	(I -LOQ) (0.00059%)	(II- ≥10LOQ) (0.01370%)
	Mean % Recovery (Accuracy)	102.30	103.50	Mean % Recovery (Accuracy)	104.06	106.10
	Overall Mean % Recovery (Accuracy)	102.90		Overall Mean % Recovery (Accuracy)	105.08	
	Acceptable Limit %	75 - 125		Acceptable Limit %	75 - 125	
Interference/ Specificity	No interference			No interference		
LOQ	mg/L (based on S/N ratio)	0.40		mg/L (based on S/N ratio)	0.40	
	% w/w (based on % recovery)	0.00078		% w/w (based on % recovery)	0.00059	
LOD	mg/L (based on S/N ratio)	0.15		mg/L (based on S/N ratio)	0.15	
Comment	The analytical method for the determination of Prothioconazole-Desthio in the plant protection product GPF515 / FGE03 with a LOQ of 0.00078%w/w was accurately validated covering specificity, linearity, precision and recovery (accuracy) according with guideline SANCO/3030/99 rev.5.			The analytical method for the determination of Toluene in the plant protection product GPF515 / FGE03 LOQ of 0.00059%w/w was accurately validated covering specificity, linearity, precision and recovery (accuracy) according with guideline SANCO/3030/99 rev.5.		

Conclusion

Acceptable analytical methods have been validated for the determination of relevant impurities Prothioconazole-desthio and Toluene with LOQ of 0.00078% w/w and 0.00059% w/w respectively in the formulation GPF515 / FGE03 and meet 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)

Under current EU legislation, analytical methods for the determination of formulants are not required. None of the formulants are of toxicological concern. Please refer to Part C for additional information.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

The CIPAC analytical method 673 is available for the determination of boscalid. According to CIPAC 673, boscalid is determined by reversed phase high performance liquid chromatography using UV detection at 260 nm and external standardisation. The method is usable for TC, WG, SE, SC-formulations.

The CIPAC analytical method 745 is available for HPLC determination of prothioconazole in TC, EC, FS and SC formulations.

No applicable CIPAC methods are available for the determination of boscalid and prothioconazole in WG formulations.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

Active substance: boscalid

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

Table 5.2.2-1 Validated methods for the generation of pre-authorization data

Component of residue definition: boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Tomato, lemon, grain oilseed rape (Residues)	Primary (DFG S19)	0.01 mg/kg mg/kg (Tomato, lemon, grain) 0.02 mg/kg (oilseed rape)	GC-MS	Weeren R.D. and Pelz S., 1999 / EU agreed (DAR 2002) 1999/11461
	Confirmatory (if required)	-		
Apple, sour cherry, grapes, strawberry, carrot, onion, tomato, broccoli, cabbage, leek, dwarf beans, oilseed rape (Residues)	ILV of DFG S19	0.01 (white cabbage, lettuce) 0.02 mg/kg (oilseed rape) 0.05 mg/kg (hops)	GC-MS	Reichert N., 2001/ EU agreed (DAR 2002) 2000/1014886
	Confirmatory (if required)	-		
White cabbage, lettuce oilseed rape hops (Residues)	Primary (DFG S19)	0.01 mg/kg	GC-MS	Class T., 2001 / EU agreed (DAR 2002) 2000/1017227
	Confirmatory (if required)	-		
Apple, sour cherry, grapes, strawberry, carrot, onion, tomato, broccoli, cabbage, leek, dwarf beans, oilseed rape (Residues)	Primary (445/0)	0.05 mg/kg (Apple, sour cherry, grapes, strawberry, carrot, onion, tomato, broccoli, cabbage, leek, dwarf beans, oilseed rape)	LC-MS/MS	Funk H. und Mackenroth Ch., 2000 / EU agreed (DAR 2002) 2000/1012404
	Confirmatory (if required)	-		
Wheat grains, wheat straw, lettuce, whole orange, oilseed rape seeds (Residues)	Primary	0.01 mg/kg	LC-MS/MS	Rastogi P.K., 2022 ^a / New study not previously reviewed S22-06799 KCP 5.1.2/01 Used in studies: S22-01574 and S23-00040 and (please refer to dRR part B7, KCA 6.3.1/01; KCA 6.5.2/01 (CP 7.2.3 and (CP 7.2.5); KCA 6.3.1/02 (CP 7.2.3))
	Confirmatory (if required)	Not required (two mass transitions)		
Animal matrices (Residues)	Primary (DFG S19)	0.01 mg/kg (milk) 0.025 mg/kg (muscle, fat,	GC-ECD	Class, T., 2001 / EU agreed (DAR 2002) 2000/1017227

Component of residue definition: boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
		kidney, liver, egg)		
	Confirmatory (if required)	Not required (two mass transitions)		
Animal matrices (Residues)	DFG S19	0.01 mg/kg (milk) and 0.025 mg/kg (liver)	GC-ECD	Kampke-Thiel, K., 2001 / EU agreed (DAR 2002) 2000/1017226
Animal matrices (honey) (Residues)	Primary	0.01 mg/kg (honey)	GC-MS/MS	Sahvorost, N., 2022 / New study not previously reviewed UPL/2022/0354 S22-00776 KCP 5.1.2/03 Used in study: S22-00761 (please refer to dRR part B7, KCA 6.10/01 (CP 7.2.7))
Animal matrices (Residues)	Primary (471/0)	0.01 (milk, cream, eggs) and 0.025 (muscle liver, kidney, fat)	LC-MS-MS	Grosshans, 2001/ EU agreed (DAR 2002) 2000/1017223
Water (Environmental fate)	Primary (411)	0.05 mg/kg	GC-MS	Keller, W., 1998 / EU agreed (DAR 2002) 1998/10922
	Primary (411)	0.05 mg/kg	GC-MS	Grote, C., 2003 / EU agreed (Addendum 2006) 2003/1000976
	Primary (411/0)	0.05 mg/kg	GC-MS	Grote, Ch., 2001/ EU agreed (DAR 2002) 2001/1008955
	Primary (411/0)	0.05 mg/kg	GC-MS	Grote, C., 2003 / EU agreed (Addendum 2006) 2003/1000975
	Confirmatory (if required)	-		
Soil (Environmental fate)	Primary (408/1)	0.01 mg/kg	GC-MS	Keller, W., 1998 / EU agreed (DAR 2002) 1998/11314
	Primary (408/1)	0.01 mg/kg	GC-MS	Grote, C. 2003 / EU agreed (Addendum 2006) 2003/1000977
	Confirmatory (if required)	-		
Air	Primary (460)	1.5 µg/m ³	GC-MS	Zangmeister, W., 2000 / EU agreed (DAR 2002) 2000/1014992
	Confirmatory (if required)	-		
Test (Ecotoxicology)	Primary	(1xAAP media) Boscalid: 196.669 µg/kg (Reconstituted water) Boscalid: 250.306 µg/kg	LC-MS/MS	Mahera, N., 2023 / New study not previously reviewed 228-2-13-33021 (UPL/2022/21191) Used in studies: 502-3-07-33000 – Daphnia and 501-3-07-32999 – algae KCP 5.1.2/04

Component of residue definition: boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	0.1 g test item/L (corresponding to 20.4 mg boscalid/L)	HPLC-UV	Venturi, S., 2024 / New study not previously reviewed BT073_23 (UPL/2023/1235) (Analytical phase, KCP 10.3.1.3/01) KCP 5.1.2/05
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	1.1 g test item/L (corresponding to 9.68 mg Boscalid/L after dilution by factor 40)	HPLC-UV	Knautz, T., Horstmann, C., 2023 / New study not previously reviewed 176531136 (UPL/2023/1209) (Analytical phase, KCP 10.3.1.2/01) KCP 5.1.2/06
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	<u>Contact test:</u> 7.5 g test item/L (corresponding to 13.2 mg Boscalid/L after dilution by factor 200) <u>Oral test:</u> 1 g test item/L (corresponding to 7.0 mg Boscalid/L after dilution by factor 50)	HPLC-UV	Chwiesko, D., Horstmann, C., 2023 / New study not previously reviewed 176531105 (UPL/2023/1279) (Analytical phase, KCP 10.3.1.1/02) KCP 5.1.2/07
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250)	HPLC-UV	Stürtz, S., Horstmann, C., 2023a / New study not previously reviewed 176531087 (UPL/2023/0743) (Analytical phase, KCP 10.6.2/01) KCP 5.1.2/08
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250)	HPLC-UV	Stürtz, D., Horstmann, C. 2023b / New study not previously reviewed 176531086 (UPL/2023/0742) (Analytical phase, KCP 10.6.2/02) KCP 5.1.2/09
	Confirmatory (if required)	-		

Table 0-5-4 5.2.2-2: Validated methods for the generation of pre-authorization data

Component of residue definition: M510F01				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Animal matrices (Residues)	Primary (DFG S19)	00.01 mg/kg (milk) 0.025 mg/kg (muscle, fat, kidney, liver, egg).01 mg/kg (milk) and 0.025 mg/kg (others)	GC-ECD	Class, T., 2001 / EU agreed (DAR 2002) 2000/1017227
	Confirmatory (if required)	-		
Animal matrices (Residues)	Primary (DFG S19)	0.01 mg/kg (milk) and 0.025 mg/kg (others)	GC-ECD	Kampke-Thiel, K., 2001 / EU agreed (DAR 2002) 2000/1017226
milk, cream, egg, muscle, liver, kidney, fat (Residues)	Primary (471/0)	0.01 mg/kg (milk, cream, eggs) and 0.025 mg/kg (others)	LC-MS/MS	Grosshans, F., 2001/ EU agreed (DAR 2002) 2000/1017223
milk, liver (cow) (Residues)	Primary (476/0)	0.01 mg/kg (milk) and 0.05 mg/kg (liver)	GC-MS	Fabian E., 2001 / EU agreed (DAR 2002) 2000/1017224

Active substance: prothioconazole

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

Table 0-5-4 5.2.2-3: Validated methods for the generation of pre-authorization data

Component of residue definition: prothioconazole*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants (Residues) Wheat (whole plant, grain, straw)	Primary	0.01 mg/kg (wheat and barley grain) 0.05 mg/kg (wheat and barley straw and green material)	HPLC-MS/MS	Heinemann 2000a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /01) (Bayer method No. 00598)
	Confirmatory (if required)	-		
Wheat Grain, Wheat Straw and green material, Barley Grain, Barley Straw and green material, Rape seed, Rape straw, pods and green material (Residues)	Primary	0.01 mg/kg (wheat and barley grain) 0.05 mg/kg (wheat and barley straw and green material) 0.01 mg/kg (rape seed) 0.05 mg/kg (rape straw, pods and green material)	HPLC-MS/MS	Heinemann 2000b/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /02) (Bayer method No. 00598/M001)
	Confirmatory (if required)	-		
Wheat Grain, Wheat Straw and green material, Barley Grain,	Primary	0.01 mg/kg (wheat and barley grain) 0.05 mg/kg (wheat and barley straw and	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /03) (Bayer method No. 00647)

Component of residue definition: prothioconazole*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Barley Straw and green material, Barley Brewing Malt, Rape seed, Rape straw, pods and green material (Residues)		green material) 0.02 mg/kg (Barley Brewing Malt) 0.01 mg/kg (rape seed) 0.05 mg/kg (rape straw, pods and green material)		
	Confirmatory (if required)	-		
Tomato and orange fruit, Wheat grain, Wheat Straw and green material, Rape seed	Primary (DFG S19)	0.02 mg/kg (Tomato and orange fruit, Wheat grain, Rape seed) 0.05 mg/kg (wheat straw and green material)	GC/MS	Weeren, Pelz 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /06) (Bayer method No. 00086/ M033, DFG Method S19)
	Confirmatory (if required)	-		
Tomato fruit, Wheat grain,	ILV ~~LV~~ of DFG S19	0.02 mg/kg (Tomato fruit, Wheat grain)	GC/MS	Class 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /07) (Bayer method No. 00086/ M033, DFG Method S19, ILV)
	Confirmatory (if required)	-		
Plants (Residues)	Primary and confirmatory*	0.005 mg/kg (prothioconazole-desthio) 0.005 mg/kg (hydroxy metabolites of prothioconazole-desthio) 0.005-0.05 mg/kg (TDMs)	LC-MS/MS	Pearson J. 2022/ New study not previously reviewed Report No. QG/20/011, KCP 5.1.2/10, KCP 5.2/04 10 Used in study QG/21/003 (please refer to dRR part B7; KCA 6.10/02 (CP 7.3.7))
Plants (Residues)	Primary and confirmatory*	oilseed rape seeds, field beans (dried), barley grain, barley forage, cucumber, orange, oilseed rape forage, wheat forage, wheat straw, wheat grains and barley straw: 0.005 mg/kg (hydroxy metabolites of prothioconazole-desthio)	LC-MS/MS	Giancola D. 2023/ New study not previously reviewed Report No. QG/22/001, KCP 5.1.2/11
Plants (Residues)	Primary and confirmatory*	Oilseed Rape: 0.005 mg/kg (TRZ) 0.005 mg/kg (TAA) 0.1 mg/kg (TA) 0.01 mg/kg (TLA)	LC-MS/MS	Phipps B. 2024/ New study not previously reviewed Report No. QG/20/012, KCP 5.1.2/12 Used in study QG/21/002 (please refer to dRR part B7; KCA 6.1/03 (CP 7.3.1))
Animal matrices (honey)	Primary and confirmatory	0.005 mg/kg (prothioconazole-	HPLC-MS/MS	Hitchens J. 2023/ New study not previously reviewed

Component of residue definition: prothioconazole*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
		desthio) 0.005 mg/kg (hydroxy metabolites of prothioconazole- desthio)		Report No. QG/21/009 Used in study QG/21/003 (please refer to dRR part B7, KCA 6.10/02 (CP 7.3.7)) KCP 5.2/14
Animal matrices (honey)	Primary and confirmatory	0.01 mg/kg (TDMs)	LC-DMS-MS/MS	Lebrun F., 2024/ New study not previously reviewed Report No. 644-2023 KCP 5.1.2/13
Milk, meat, offal (Residues)	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /04) (Bayer method No. 00655)
	Confirmatory (if required)	-		
Milk (Residues)	Primary	0.004 mg/kg	HPLC-MS/MS	Heinemann 2001c/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /05) (Bayer method No. 00655/M001)
	Confirmatory (if required)	-		
Milk, meat, offal (Residues)	ILV	0.01 mg/kg Meat, offal) 0.004 mg/kg (milk)	HPLC-MS/MS	Dubey 2001/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /08) (Bayer method No. A-14-01-01, ILV)
	Confirmatory (if required)	-		
Soil (Environmental fate)	Primary	0.006 mg/kg	HPLC-MS/MS	Schramel, 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.2.1 /01) (Bayer method No. 00610)
	Primary	0.01 mg/kg	GC/MS	Steinhauer, 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.2.1 /03) (Bayer method No. 00086/M038) DFG Method S 19
	Confirmatory (if required)	-		
Water (Environmental fate)	Primary	6 µg/L	HPLC-UV	Sommer, 1999 / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.2.1 /02) (Bayer method No. 00586)
	Primary	10 µg/L (JAU6476-S methyl)	HPLC-UV	Sommer, H., 2001a / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A,4.2.3.1 /02) (Bayer method No. 00699)
	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole- desthio)	HPLC-MS/MS	Sommer, H., 2001b / EU agreed /DAR Addendum 6 (B.5.2 -2006, II A,4.2.3.1 /03) (Bayer method No. 00684)
	Confirmatory	-		

Component of residue definition: prothioconazole*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	(if required)			
Air	Primary	0.015 µg/L (prothioconazole) 0.0006 µg/L (prothioconazole- desthio)	HPLC-MS/MS	Maasfeld, 2002a and 2002b / EU agreed /DAR Addendum 6 (B.5.2 - 2006, II A, 4.2.4.1 /01, II A, 4.2.4.1 /02) (Bayer method No. 00724 and No. 00731)
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	(1xAAP media) Prothioconazole: 109.571 µg/kg (Reconstituted water) Prothioconazole: 139.454 µg/kg	LC-MS/MS	Mahera, N., 2023 / New study not previously reviewed 228-2-13-33021 (UPL/2022/21191) Used in studies: 502-3-07-33000 – Daphnia and 501-3-07-32999 – algae KCP 5.1.2/04
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	0.1 g test item/L (corresponding to 35.5 mg prothioconazole/L)	HPLC-UV	Venturi, S., 2024 / New study not previously reviewed BT073_23 (UPL/2023/1235) (Analytical phase) KCP 5.1.2/05
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	1.1 g test item/L (corresponding to 40 5.59 mg Prothioconazole/L after dilution by factor 40)	HPLC-UV	Knautz, T., Horstmann, C., 2023 / New study not previously reviewed 176531136 (UPL/2023/1209) (Analytical phase) KCP 5.1.2/06
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	<u>Contact test:</u> 7.5 g test item/L (corresponding to 7.6 mg Prothioconazole/L after dilution by factor 200) <u>Oral test:</u> 1 g test item/L (corresponding to 4.1 mg Prothioconazole/L after dilution by factor 50)	HPLC-UV	Chwiesko, D., Horstmann, C., 2023 / New study not previously reviewed 176531105 (UPL/2023/1279) (Analytical phase, KCP 10.3.1.1.1/02) KCP 5.1.2/07
	Confirmatory (if required)	-		
Test solution (Ecotoxicology)	Primary	12 g test item/L corresponding to Prothioconazole/L (diluted by factor 250)	HPLC-UV	Stürtz, S., Horstmann, C., 2023a / New study not previously reviewed 176531087 (UPL/2023/0743) (Analytical phase, KCP 10.6.2/01) KCP 5.1.2/08
	Confirmatory (if required)	-		

Component of residue definition: prothioconazole*				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	(if required)			
Test solution (Ecotoxicology)	Primary	12 g test item/L corresponding to Prothioconazole/L (diluted by factor 250)	HPLC-UV	Stürtz, D., Horstmann, C. 2023b / New study not previously reviewed 176531086 (UPL/2023/0742) (Analytical phase, KCP 10.6.2/02) KCP 5.1.2/09
	Confirmatory (if required)	-		

*The components of the residue definition are:

- 1) 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);
 - 2) Triazole alanine (TA) and triazole lactic acid (TLA);
 - 3) Triazole acetic acid (TAA);
 - 4) 1,2,4-triazole (1,2,4-T).
- (EFSA Scientific Report (2007) 106, 1-98 and Prothioconazole SANCO/3923 /07 - final 10 December 2007 26 January 2021)

zRMS comments:

The Applicant submitted a number of methods for analysis of residues of boscalid and prothioconazole for the generation of pre-authorization data. The analytical methods were successfully validated and accepted. 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 substances and relevant impurities in the plant protection product are submitted under point 5.2.1.

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

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

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

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	Boscalid	0.01* mg/kg 1.50 mg/kg (pears) 3.00 mg/kg (fresh beans and peas without pods, plums) 5.00 mg/kg (fresh beans and peas with pods, stone fruits)	Reg. (EU) 2022/1324
Plant, high acid content		0.01* mg/kg 5.00 mg/kg (table and wine grapes, kiwi)	Reg. (EU) 2022/1324
Plant, high protein/high starch content (dry commodities)		0.15 mg/kg (cereals) 0.05 mg/kg (chestnuts)	Reg. (EU) 2022/1324

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high oil content		0.01* mg/kg 1 mg/kg (flax, oilseed rape)	Reg. (EU) 2022/1324
Plant, difficult matrices (hops, spices, tea)		0.01* mg/kg 0.05 mg/kg	Reg. (EU) 2022/1324
Muscle	Boscalid in muscle, fat, milk and eggs and the sum of boscalid and its hydroxy metabolite M510F01 (free and conjugated), expressed as boscalid, in liver and kidney	0.01* mg/kg	Reg. (EU) 2022/1324
Milk		0.02 mg/kg	Reg. (EU) 2022/1324
Eggs		0.01* mg/kg	Reg. (EU) 2022/1324
Fat		0.01 mg/kg 0.07 mg/kg	Reg. (EU) 2022/1324
Liver, kidney		0.01 mg/kg 0.05* mg/kg	Reg. (EU) 2022/1324
Honey	Boscalid	0.15 mg/kg	Reg. (EU) 2022/1324
Soil (Ecotoxicology)	Boscalid	0.05 mg/kg	Common limit
Drinking water (Human toxicology)	Boscalid and metabolite M510F049	0.1 µg/L	General limit for drinking water
Surface water (Ecotoxicology)	Boscalid	NOEC: 0.125 µg/L	The value refers to study on fish. SANCO/3919 /2007-rev. 5
Air	Boscalid	30 µg/m ³	AOEL sys: 0.1 mg/kg bw/d SANCO/3919 /2007-rev. 5
Tissue (meat or liver)	Not required as the active substance is not toxic or very toxic	0.01 (*) animal mg/kg	Not classified as T / T+ SANTE/2020/12830, Rev.2
Body fluids	Boscalid and metabolite M510F01	Not required 0.01 mg/L	Not classified as T / T+ SANTE/2020/12830, Rev.2

(*) Limit of analytical determination

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

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

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

Component of residue definition: Boscalid				
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	GC-MS	Weeren and Pelz, 1999 / EU agreed / DAR 2002 DocID 1999/11461
	ILV	0.01 mg/kg	GC-MS	Reichert, 2001 / EU agreed / DAR 2002 DocID 2000/1014886
	Confirmatory (if required)	Not required as ILV is highly selective		
	Primary	0.05 mg/kg	HPLC-MS/MS	Funk and Mackenroth, 2001/ EU agreed / DAR 2002

Component of residue definition: Boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
				DocID 2000/1012404
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (lettuce)	0.01 mg/kg	HPLC-MS/MS	Rastogi, P.K., 2022 / New study not previously reviewed S22-00980, KCP 5.2/01
	ILV	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2022 / New study not previously reviewed S22-05632, KCP 5.2/02
	Confirmatory (if required)	Not necessary (two mass transitions)		
High acid content	Primary	0.01 mg/kg	GC-MS	Weeren and Pelz, 1999 / EU agreed / DAR 2002 DocID 1999/11461
	ILV	0.01 mg/kg	GC-MS	Reichert, 2001 / EU agreed / DAR 2002 DocID 2001/1014886
	Confirmatory (if required)	Not required as ILV is highly selective		
	Primary	0.05 mg/kg	HPLC-MS/MS	Funk and Mackenroth, 2001/ EU agreed / DAR 2002 DocID 2000/1012404
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	not necessary (two mass transitions)		
	Primary (whole orange)	0.01 mg/kg	HPLC-MS/MS	Rastogi, P.K., 2022 / New study not previously reviewed S22-00980, KCP 5.2/01
	ILV	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2022 / New study not previously reviewed S22-05632, KCP 5.2/02
	Confirmatory (if required)	Not necessary (two mass transitions)		
High oil content	Primary	0.02 mg/kg (rapeseed oil)	GC-MS	Weeren and Pelz, 1999 / EU agreed / DAR 2002 DocID 1999/11461
	ILV	0.02 mg/kg	GC-MS	Reichert, 2001 / EU agreed / DAR 2002 DocID 2001/1014886
	Confirmatory (if required)	Not necessary as ILV is highly selective		

Component of residue definition: Boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Primary	0.05 mg/kg	HPLC-MS/MS	Funk and Mackenroth, 2001/ EU agreed / DAR 2002 DocID 2000/1012404
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (oilseed rape seeds)	0.01 mg/kg	HPLC-MS/MS	Rastogi, P.K., 2022 / New study not previously reviewed S22-00980, KCP 5.2/01
	ILV	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2022 / New study not previously reviewed S22-05632, KCP 5.2/02
	Confirmatory (if required)	Not necessary (two mass transitions)		
High protein/high starch content (dry)	Primary	0.01 mg/kg	GC-MS	Weeren and Pelz 1999 / EU agreed / DAR 2002 DocID 1999/11461
	ILV	Not necessary as ILV is performed in high water and high oil content		
	Confirmatory (if required)	Not necessary as the method is highly selective		
	Primary (wheat grains and straw)	0.01 mg/kg	HPLC-MS/MS	Rastogi, P.K., 2022 / New study not previously reviewed S22-00980, KCP 5.2/01
	ILV	0.01 mg/kg	HPLC-MS/MS	Gustloff, C., 2022 / New study not previously reviewed S22-05632, KCP 5.2/02
	Confirmatory (if required)	Not necessary (two mass transitions)		
Difficult (if required, depends on intended use)	Primary	0.05 mg/kg (hops, recovery = 63 %)	HPLC-MS/MS	Reichert, 2001 / EU agreed / DAR 2002 DocID 2001/1014886
	Not required as hops are not included in the intended uses			

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

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Investigations on the extractability of 14C-BAS 510 F residues from plant matrices; Bross, M., 2001 Study code 73479 2001/1001739 GLP

	Method for products of plant origin
	Unpublished MET2001-268 which is already peer-reviewed in the DAR for the evaluation of boscalid (DAR, 2002).
Not required, because:	As the extraction procedure used in the residue analytical method 445/0 and the multi residue method S19 slightly deviates from those used in the metabolism studies, ¹⁴ C nicobifen treated plant material was extracted according to these methods and the results thereof were compared. Purpose of this study is to verify the extraction efficiency of the “cold methods” in extracting the total radioactive residue (TRR). The extraction efficiency is proven sufficiently for commodities with high water content, with high oil content, for dry and acidic crops.

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

zRMS comments:

According to the EFSA Journal 2014;12(7):3799:

Methods for enforcement of residues in food of plant origin

“During the peer review under Directive 91/414/EEC, an analytical method using GC-MS and its ILV was evaluated and validated for the determination of boscalid in plant matrices with an LOQ of 0.01 mg/kg in high water content (tomato), 0.02 mg/kg in high fat content (oilseed rape), and 0.01 mg/kg in acidic (lemon) and dry commodities (wheat grain) (Germany, 2002). The method is however not highly specific according to SANCO/825/00 rev 8.1 (EC, 2010b).

In addition, an analytical method using HPLC-MS/MS was evaluated and validated for the determination of boscalid in plant matrices with an LOQ of 0.05 mg/kg in high water (apple, sour cherry, strawberry, carrot, onion, tomato, broccoli, white cabbage, leek, dwarf bean), high acid (grape) and high fat content (oilseed rape) commodities (Germany, 2002; FAO, 2006).

The multi-residue QuEChERS method in combination with HPLC-MS/MS, as described by CEN (2008), is also reported for analysis of parent boscalid with an LOQ of 0.01 mg/kg in dry commodities, high water content, high fat content and acidic commodities.

Hence it is concluded that boscalid can be enforced in food of plant origin with an LOQ of 0.01 mg/kg in high water content, high fat content, dry and acidic commodities.

No analytical method is available for hops, spices and herbal infusions. Considering that analytical methods are validated in four matrix groups and that dilutions of extracts before analysis should reduce the analysis difficulties inherent to those commodities, it is likely that validated methods presented above can be used to enforce the high residue levels observed in hops, spices and herbal infusions. Nevertheless, full validation data is in principle still required.”

It should be noted that the ILV method of Reichert N. (2001 BASF DocID 2000/1014886) was validated in white cabbage (high water content commodity), rape seed (high oil content commodities), hops (no group) and lettuce (high water content commodity). According to the SANTE/2020/12830, Rev.2, 14. February 2023 *If the primary method is identical for all matrix groups, it is sufficient to perform the ILV for commodities of two of these groups, one of them with high water content.*

The new multi-residue method QuEChERS and its ILV for determination of residues of boscalid was fully validated on in/on five different matrices of plant origin i.e. wheat grains, wheat straw, lettuce, whole orange and oilseed rape seeds according to SANTE/2020/12830, rev.1 with LOQ of 0.01 mg/kg (Rastogi, T., 2022b, Study Number: S22-00980; ILV: Gustloff, C., 2022, Study Number: S22-05632).

The lack of an analytical method for hops, spices and herbal infusions has been identified during review of the Maximum Residue Levels (EFSA Journal 2014;12(7):3799) (data gap). **This data gap should be addressed during the renewal of boscalid.**

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

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

referred to Appendix 2.

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

Component of residue definition: Boscalid and metabolite M510F01				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.01 mg/kg	GC-MS	Class, T., 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017227
	ILV	0.01 mg/kg	GC-MS	Kampke-Thiel, 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017226
	Confirmatory (if required)	Not necessary as the ILV is highly selective		
	Primary	0.01 mg/kg	HPLC-MS/MS	Grosshans, F, 2001 / EU agreed / DAR 2002 BASF-method 471/0 2000/1017223
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (only for bound metabolite M510F53)	0.01 mg/kg	GC-MS	Fabian, E. / 2001 / EU agreed / DAR 2002 BASF-method 476 2000/1017224
	Primary (boscalid only)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV (boscalid only)	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
Eggs	Primary	0.025 mg/kg	GC-MS	Class, T., 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017227
	ILV	Not necessary as ILV is provided for milk and liver		
	Confirmatory (if required)	Not necessary as the method is highly selective		
	Primary	0.01 mg/kg	HPLC-MS/MS	Grosshans, F, 2001 / EU agreed / DAR 2002 BASF-method 471/0 2000/1017223
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is		

Component of residue definition: Boscalid and metabolite M510F01				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
		required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (boscalid only)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
Muscle	Primary	0.025 mg/kg	GC-MS	Class, T., 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017227
	ILV	Not necessary as ILV is provided for milk and liver		
	Confirmatory (if required)	Not necessary as the method is highly selective		
	Primary	0.025 mg/kg	HPLC-MS/MS	Grosshans, F, 2001 / EU agreed / DAR 2002 BASF-method 471/0 2000/1017223
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (boscalid only)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
Fat	Primary	0.025 mg/kg	GC-MS	Class, T., 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017227
	ILV	Not necessary as ILV is provided for		

Component of residue definition: Boscalid and metabolite M510F01				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
		milk and liver		
	Confirmatory (if required)	Not necessary as the method is highly selective		
	Primary	0.025 mg/kg	HPLC-MS/MS	Grosshans, F, 2001 / EU agreed / DAR 2002 BASF-method 471/0 2000/1017223
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (boscalid only)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
Kidney, liver	Primary	0.025 mg/kg	GC-MS	Class, T., 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017227
	ILV	0.025 mg/kg	GC-MS	Kampke-Thiel, 2001 / EU agreed / DAR 2002 DFG S19 method 2000/1017226
	Confirmatory (if required)	Not necessary as the ILV is highly selective		
	Primary	0.025 mg/kg	HPLC-MS/MS	Grosshans, F, 2001 / EU agreed / DAR 2002 BASF-method 471/0 2000/1017223
	ILV	As a vast number of validated enforcement methods using QuEChERS approach is available from the EURL (European Reference Laboratories for Residues of Pesticides) Data Pool, no additional ILV (independent laboratory validation) is required.		
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (only for bound metabolite M510F53)	0.05 mg/kg	GC-MS	Fabian, E., 2001 / EU agreed / DAR 2002 BASF-method 476 2000/1017224
	Primary (boscalid, and M510F01)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed

Component of residue definition: Boscalid and metabolite M510F01				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
				UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
	Primary (M510F52 and M510F53 for liver only)	0.01 mg/kg	HPLC-MS/MS	Rastogi, T., 2023 / New study not previously reviewed UPL/2022/2043 S22-00981 KCP 5.2/03
	ILV	0.01 mg/kg	HPLC-MS/MS	Spielvogel Ch., & Timmann A., 2023 / New study not previously reviewed UPL/2022/05633 S22-05633 (EAG-2202V) KCP 5.2/04
	Confirmatory (if required)	Not necessary (two mass transitions)		
Honey	Primary	0.01 mg/kg	GC-MS/MS	Sahvorost, N., 2022 / New study not previously reviewed UPL/2022/0354 S22-00776
	ILV	0.01 mg/kg	GC-MS/MS	Rastogi, T., 2022 / New study not previously reviewed UPL/2022/0845 S22-00783
	Confirmatory (if required)	Not necessary (two mass transitions)		

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 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	Extraction efficiency has been assessed during the Annex I inclusion process (DAR 2002). The extraction solvent used in the residue analytical method 471/0 (methanol) as well as in the multimethod approach DFG S19 removed comparable amounts of residues than the metabolism extraction scheme.

For the detailed evaluation of (additional) studies on extraction efficiency please refer to Appendix 2.

zRMS comments:

According to the EFSA Journal 2014;12(7):3799:

Methods for enforcement of residues in food of animal origin

“During the peer review under Directive 91/414/EEC, an analytical method using GC-ECD, confirmed by GC-MS, and its ILV were evaluated and validated for the determination of boscalid and its metabolite M510F01 (free and conjugated) in food of animal origin with an LOQ of 0.01 mg/kg for each compound in milk and 0.025 mg/kg for each compound in meat, fat, liver, kidney and eggs (Germany, 2002). The method should be able to analyse

the conjugates of the metabolite but the hydrolysis step has not been validated. Moreover, the method is not highly specific according to SANCO/825/00 rev 8.1 (EC, 2010b).

Nevertheless, an analytical method using HPLC-MS/MS was also evaluated and validated for the determination of boscalid and its metabolite M510F01 (free and conjugated) in animal matrices with an LOQ of 0.01 mg/kg for each compound in milk, cream and eggs, and 0.025 mg/kg for each compound in muscle, fat, kidney and liver. The method is able to analyse the conjugates of the metabolite and the hydrolysis step is considered as validated. Hence it is concluded that boscalid can be enforced in food of animal origin with an LOQ of 0.01 mg/kg in milk and eggs, and an LOQ of 0.025 mg/kg in muscle and fat. Boscalid and its metabolite M510F01 (free and conjugated) can be enforced in liver and kidney with an LOQ of 0.025 mg/kg for each compound. Further validation of the hydrolytic step dissociating the conjugates of metabolite M510F01 should be required for GC-ECD method. However, considering the hydrolysis step was validated for another analytical method, this information is considered as desirable only (minor deficiency)."

Three new analytical methods and ILV for determination of residues of boscalid, M510F01, M510F52 and M510F53 were fully validated on in/on matrices of animal origin such as bovine meat (as muscle), bovine fat, milk, poultry's eggs, bovine liver and bovine kidney according to SANTE/2020/12830, rev.1 with LOQ of 0.01 mg/kg in each matrices (Rastogi, T. 2023, Study No. S22-00981, ILV: Spielvogel Ch., Timmann, A., 2023; Study No. S22-05633 (EAG-2202V)).

Additionally, analytical method and its ILV for determination of boscalid in honey with LOQ of 0.01 mg/kg is available. The method is accepted.

More details please see in Appendix 2.

No additional data are required.

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

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

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

Component of residue definition: Boscalid			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg	GC-MS	Keller, W., 1998a / EU agreed / DAR 2002 BASF-method 408/1 1998/11314 Grote C., 2003 / EU agreed / ADD 2006 2003/1000977
Confirmatory	Not necessary as the method is highly selective		

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

zRMS comments:

The GC-MS BASF-method 408/1 is available for determination of boscalid with LOQ of 0.01 mg/kg in soil. No additional data are required.

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

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

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

Component of residue definition: Boscalid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water / ground water	Primary	0.05 µg/L	GC-MS	Grothe, Ch., 2001 / EU agreed / DAR 2002 BASF-method 411/0 2001/1008955 Grote C., 2003 / EU agreed / ADD 2006 2003/1000975
	Primary	0.1 µg/L	LC-MS/MS	Rastogi, T., 2022 / New study not previously reviewed UPL/2022/2227 S22-00979
	ILV	0.1 µg/L	LC-MS/MS	Kaiser, M., 2023 / New study not previously reviewed UPL/2022/21136 S22-01028
	Confirmatory	Not required (two mass transitions)		
Surface water	Primary	0.05 µg/L	G-MS	Keller, W., 1998 / EU agreed / DAR 2002 BASF-method 411 1998/10922 Grote C., 2003 / EU agreed / ADD 2006 2003/1000976
	Confirmatory	Not necessary as the method is highly selective		
	Primary	0.1 µg/L	LC-MS/MS	Rastogi, T., 2022 / New study not previously reviewed UPL/2022/2227 S22-00979

Table 5.3-8: Validated methods for water (boscalid metabolites M510F47, M510F49 and M510F64)

Component of residue definition: M510F47, M510F49, M510F64				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Surface water, groundwater	Primary & Confirmatory (2 mass transitions)	0.1 µg/L	LC-MS/MS	Rastogi, T., 2022 / New study not previously reviewed UPL/2022/2227 S22-00979
Ground water, Drinking water	ILV	0.1 µg/L	LC-MS/MS	Kaiser, M., 2023 / New study not previously reviewed UPL/2022/21136 S22-01028

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

zRMS comments:

The Applicant provided additional analytical method for the determination of boscalid and metabolites M510F47, M510F49 and M510F64 in groundwater and surface water.
The analytical method of Rastogi, T. (2022) has been successfully validated and fulfils the requirements with regard to specificity, linearity, limit of quantification, recoveries and repeatability for the analysis of BAS 510 F

(boscalid) and boscalid metabolites M510F47, M510F49 and M510F64 in groundwater and surface water with LOQ of 0.1 µg/kg using LC-MS/MS. The method is independently validated (Kaiser, M., 2023). No additional data are required.

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

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

Table 5.3-9: Validated methods for air (if appropriate)

Component of residue definition: Boscalid			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	1.5 µg/m ³	GC-MS	Zangmeister, W., 2000 / EU agreed (DAR 2002) 2000/1014992
Confirmatory	Not necessary as the method is highly selective		

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

zRMS comments:

The GC-MS method is available for determination of boscalid with LOQ of 1.5 µg/m³ in air. No additional data are required.

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

An overview on the acceptable methods and possible data gaps for analysis of boscalid 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 5.3-10: Methods for body fluids and tissues (if appropriate)

Component of residue definition: boscalid and metabolite M510F01			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg L	An entry is missing here LC-MS/MS	Rastogi, T., 2022 / New study not previously reviewed UPL/2022/2054 S22-00982
Confirmatory	Not required (two mass transitions)		

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

A new analytical method (Rastogi, T., 2022) for the determination of boscalid and its metabolite M510F01 in/on body fluids (blood and urine) has been provided.

For the determination of residues in body tissues, we refer to the analytical methods already validated for animal matrices, as presented in section 5.3.2.3, Table 5.3-4 of the dossier. These methods are applicable to body tissues, provided that the same residue definition and MRL/limit apply.

zRMS comments:

The Applicant provided additional analytical method for the determination of boscalid and its metabolite M510F01 in/on body fluids (blood and urine).

The analytical method of Rastogi, T. (2022) has been successfully validated and fulfils the requirements with regard to specificity, linearity, limit of quantification, recoveries and repeatability for the analysis of boscalid and its metabolite M510F01 in/on body fluids (blood and urine) with LOQ of 0.01 mg/L for both analytes in both

investigated matrices using LC-MS/MS.
No additional data are required.

5.3.2.8 Other studies/ information

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

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

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	Prothioconazole: prothioconazole-desthio (sum of isomers)	0.01 mg/kg 0.02 mg/kg (potatoes) 0.1 mg/kg (carrots)	Reg. (EU) 2024/1318
Plant, high acid content		0.01* mg/kg (citrus fruits, berries and small fruits) 0.15 mg/kg (cranberries)	Reg. (EU) 2024/1318
Plant, high protein/high starch content (dry commodities)		0.01* mg/kg (legume vegetables)	Reg. (EU) 2024/1318
Plant, high oil content		0.01* mg/kg (tree-nuts table olives) 0.15 (rapeseeds/canola seeds)	Reg. (EU) 2024/1318
Plant, difficult matrices (hops, spices, tea)		0.05* mg/kg	Reg. (EU) 2024/1318
Muscle	Prothioconazole: 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 (poultry) 0.02 mg/kg (other animals)	Reg. (EU) 2024/1318
Liver, kidney		0.1 mg/kg (poultry) 0.5 mg/kg (other animals)	Reg. (EU) 2024/1318
Honey	Prothioconazole: prothioconazole-desthio (sum of isomers)	0.05 mg/kg	Reg. (EU) 2024/1318
Soil (Ecotoxicology)	Prothioconazole, Prothioconazole -desthio (M04)	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Prothioconazole, Prothioconazole-desthio (M04)	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Prothioconazole, Prothioconazole-desthio (M04)	RAC 13 µg/L (prothioconazole)	from EC50 for <i>Daphnia magna</i> (ESFA Scientific Report, 2007)
		RAC 0.334 µg/L (prothioconazole-desthio)	from NOEC for <i>Oncorhynchus mykiss</i> (ELS) (ESFA Scientific Report, 2007)
Air	Prothioconazole, prothioconazole-desthio (M04)	LOQ = 0.15 µg/m ³ (prothioconazole) LOQ = 0.0006 µg/m ³	EFSA Scientific Report (2007)

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
		(prothioconazole- desthio)	
Tissue (meat or liver)	prothioconazole-desthio (sum of isomers)	0.01 mg/kg	General limit according to SANTE/2020/12830, Rev.2
Body fluids		0.01 mg/L	General limit according to SANTE/2020/12830, Rev.2

(*) Limit of analytical determination

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

Table 5.3-12: 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	0.02 mg/kg (tomato)	DFG S19 GC-MS	Weeren, Pelz 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19)
	Primary	0.005 mg/kg (cucumber, wheat forage, barley forage)	LC-MS/MS	J. Pearson, 2022, New study not previously reviewed Report No. QG/20/011
	ILV	0.02 mg/kg (tomato)	DFG S19 GC-MS	Class 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19, ILV)
	ILV	0.005 mg/kg (cucumber)	LC-MS/MS	N. Boubakri, 2023, New study not previously reviewed Report No. S21-08354
	Confirmatory (if required)	Not required (ILV is highly selective and/or two mass transitions)		
High acid content	Primary	0.02 mg/kg (orange)	DFG S19 GC-MS	Weeren, Pelz 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19)
	Primary	0.005 mg/kg (orange)	LC-MS/MS	J. Pearson, 2022, New study not previously reviewed Report No. QG/20/011
	ILV	0.02 mg/kg	DFG S19 GC-MS	Class 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19, ILV)
		0.005 mg/kg (orange)	LC-MS/MS	N. Boubakri, 2023, New study not previously reviewed Report No. S21-08354,
	Confirmatory (if required)	Not required (ILV is highly selective and/or two mass transitions)		

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 oil content	Primary	0.02 mg/kg (Rape seed)	DFG S19 GC-MS	Weeren, Pelz 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19)
	Primary	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	J. Pearson, 2022, New study not previously reviewed Report No. QG/20/011,
	ILV	0.02 mg/kg (Rape seed)	DFG S19 GC-MS	Class 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19, ILV)
	ILV	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	N. Boubakri, 2023, New study not previously reviewed Report No. S21-08354,
	Confirmatory (if required)	Not required (ILV is highly selective and/or two mass transitions)		
High protein/high starch content (dry)	Primary	0.02 mg/kg (wheat grain)	DFG S19 GC-MS	Weeren, Pelz 2000 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19)
	Primary	0.005 mg/kg (dried field bean, wheat straw, barley straw)	LC-MS/MS	Pearson J. 2022, New study not previously reviewed Report No. QG/20/011
	ILV	0.02 mg/kg (wheat grain)	DFG S19 GC-MS	Class 2001 / EU agreed /DAR Addendum 6 (B.5.2 -2006) (Bayer method No. 00086/ M033, DFG Method S19, ILV)
	ILV	0.005 mg/kg (dried field bean)	LC-MS/MS	Boubakri N. 2023, New study not previously reviewed Report No. S21-08354
	Confirmatory (if required)	Not required (ILV is highly selective and/or two mass transitions)		
Difficult (if required, depends on intended use)	Not relevant for the intended uses of this product			

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

Table 5.3-13: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Draft Assessment Report DAR – PROTHIOCONAZOLE, July 2005, Volume 3, Annex B.5 and B7 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 metabolism 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: prothioconazole-desthio (sum of isomers)				
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	Heinemann 2001c/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /05) (Bayer method No. 00655/M001)
	ILV	0.004 mg/kg	HPLC-MS/MS	Dubey 2001/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /08) (Bayer method No. A-14-01-01, ILV)
	Confirmatory (if required)	Not required as the primary method is highly specific		
Eggs	Primary	0.01 mg/kg	LC-MS/MS	Kleinhenz D. 2023, New study not previously reviewed Report No. S21-08355
	ILV	0.01 mg/kg	LC-MS/MS	Rastogi T. 2023, New study not previously reviewed Report No. S21-08868
	Confirmatory (if required)	Not required (two mass transitions)		
Muscle	Primary	0.001 mg/kg	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /04) (Bayer method No. 00655)
	ILV	0.001 mg/kg	HPLC-MS/MS	Dubey 2001/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /08) (Bayer method No. A-14-01-01, ILV)
	Confirmatory (if required)	Not required as the primary method is highly specific		
Fat	Primary	0.001 mg/kg	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /04) (Bayer method No. 00655)
	ILV	0.001 mg/kg	HPLC-MS/MS	Dubey 2001/ EU agreed /DAR

Component of residue definition: Prothioconazole: prothioconazole-desthio (sum of isomers)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
				Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /08) (Bayer method No. A-14-01-01, ILV)
	Confirmatory (if required)	Not required as the primary method is highly specific		
Kidney, liver	Primary	0.001 mg/kg	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /04) (Bayer method No. 00655)
	ILV	0.001 mg/kg	HPLC-MS/MS	Dubey 2001/ EU agreed /DAR Addendum 6 (B.5.2 -2006, II A, 4.2.1.1 /08) (Bayer method No. A-14-01-01, ILV)
	Confirmatory (if required)	Not required as the primary method is highly specific		
Honey	Primary	0.005 mg/kg	LC-MS/MS	Hitchens J. 2023, New study not previously reviewed Report No. QG/21/009
	ILV	0.005 mg/kg	LC-MS/MS	Wagner J. 2023, New study not previously reviewed Report No. S21-08357
	Confirmatory (if required)	Not required, two mass transitions		

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:	Draft Assessment Report DAR – PROTHIOCONAZOLE, July 2005, Volume 3, Annex B.5 and B7 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.

For the detailed evaluation of (additional) studies on extraction efficiency please refer to Appendix 2.

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)

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

Table 5.3-16: Validated methods for soil (if appropriate)

Component of residue definition: Prothioconazole, Prothioconazole-desthio (M04)			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Primary	0.006 mg/kg for Prothioconazole 0.006 mg/kg for Prothioconazole-desthio	HPLC-MS/MS	Schramel, 2000 / EU agreed / DAR Addendum 6 (B.5.3.1 - 2006) (Bayer method No. 00610 (MR-643/99))
Primary	0.01 mg/kg for Prothioconazole-desthio	GC/MS	Steinhauer, 2001 / EU agreed / DAR Addendum 6 (B.5.3.1 - 2006) (Bayer method No. 00086/M038)
Primary	0.006 mg/kg (prothioconazole) 0.002 mg/kg (prothioconazole-desthio)	LC-MS/MS	Kaiser M. 2022, New study not previously reviewed Report No. S21-08358,
Confirmatory	Not necessary as the method is highly selective and/or two mass transitions		

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 valuation of new/ additional studies it is referred to Appendix 2.

Table 5.3-17: Validated methods for water (if appropriate)

Component of residue definition: Prothioconazole, Prothioconazole-desthio (M04)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Drinking water	Primary	0.1 µg/L for Prothioconazole 0.05 µg/L for Prothioconazole-desthio	HPLC-MS/MS	Sommer, H., 2001b / EU agreed / DAR Addendum 6 (B.5.3.2 - 2006, II A,4.2.3.1 /03) (Bayer method No. 00684)
	Primary	0.05 µg/L	LC-MS/MS	Kaiser D. 2022, New study not previously reviewed Report No. S21-08359
	ILV	0.05 µg/L	LC-MS/MS	Jooß S. 2023, New study not previously reviewed Report No. S21-08869
	Confirmatory	Not required as the primary method is highly specific		
Surface water	Primary	0.1 µg/L for Prothioconazole 0.05 µg/L for Prothioconazole-desthio	HPLC-MS/MS	Sommer, H., 2001b / EU agreed / DAR Addendum 6 (B.5.3.2 - 2006, II A,4.2.3.1 /03) (Bayer method No. 00684)
	Primary	0.05 µg/L	LC-MS/MS	Kaiser D., 2022, New study not previously reviewed Report No. S21-08359,
	Confirmatory	Not required as the primary method is highly specific		

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 please refer to Appendix 2.

Table 5.3-18: Validated methods for air (if appropriate)

Component of residue definition: Prothioconazole, Prothioconazole-desthio (M04)			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.015 µg/m³ for prothioconazole	HPLC-MS/MS	Maasfeld, 2002a / EU agreed / DAR Addendum 6 (B.5.3.3 - 2006, II A, 4.2.4.1 /01) (Bayer method No. 00724)
Primary		HPLC-MS/MS	(Maasfeld 2002b / EU agreed /

Component of residue definition: Prothioconazole, Prothioconazole-desthio (M04)			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
	0.0006 µg/m ³ for prothioconazole-desthio		DAR Addendum 6 (B.5.3.3 - 2006, II A, 4.2.4.1 /02) (Bayer method No. 00731)
Confirmatory	Not required as the primary method is highly specific		

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 (M04)			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.001 mg/kg	HPLC-MS/MS	Heinemann 2001a/ EU agreed /DAR Addendum 6 (B.5.2 - 2006, I A, 4.2.1.1 /04) (Bayer method No. 00655)
Primary	0.01 mg/L	LC-MS/MS	Boubakri N. 2023, New study not previously reviewed Report No. S21-08361,
Confirmatory	Not required as the primary method is highly specific		

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

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	Desai H.	2023	Validation of analytical method for determination of active ingredient content of boscalid 350 g/kg + prothioconazole 200 g/kg WG UPL/2023/0300 Jai Research Foundation 228-2-12-33016 GLP Unpublished	No	UPL
KCP 5.1.1/02	Desai H.	2024	Validation of Analytical Method for Determination of Associated Impurities of Boscalid 350 g/Kg + Prothioconazole 200 g/Kg WG UPL/2024/1238 Jai Research Foundation 228-2-12-37860 GLP Unpublished	No	UPL
KCP 5.1.2/01	Rastogi T.	2022 ^a	Validation of the Analytical Method for Determination of Boscalid in Different Matrices of Plant Origin. Study UPL/2022/2099 Eurofins Agrosience Services, EAG Laboratories GmbH S22-06799 GLP Unpublished	No	UPL
KCP 5.1.2/02	Rastogi T.	2023 ^a	Cross-Validation - Comparing Amounts of Boscalid extracted from Samples of Plant Origin with incurred Residues using three different Solvent Systems. UPL/2023/1062 Eurofins Agrosience Services, EAG Laboratories GmbH S22-00983 GLP Unpublished	No	UPL
KCP 5.1.2/03 KCP 5.2/0 ⁵	Sahvorost N.	2022	Validation of the Analytical Method for the Determination of Residues of Boscalid in Honey, Report Amendment 1, UPL/2022/0354 Eurofins Agrosience Services EcoChem GmbH S22-00776 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 5.1.2/04	Mahera N.	2023	Validation of Analytical Method for Determination of Active Ingredient Concentration and Stability of Boscalid and Prothioconazole in Matrix, following the Application of Boscalid 350 g/kg and Prothioconazole 200 g/kg WG UPL/2022/21191 Jai Research Foundation 228-2-13-33021 Used in studies: 502-3-07-33000 – Daphnia and 501-3-07-32999 - algae	No	UPL
KCP 5.1.2/05 (filled under KCP 10.3.1.3/01)	Venturi, S.	2024	Effects of Boscalid + Prothioconazole (350 + 200 g/kg – WG) on honeybees (Apis mellifera L.) 22-day larval toxicity test with repeated exposure UPL/2023/1235 BioTecnologie BT S.r.l. BT073_23 (Analytical phase, KCP 10.3.1.3/01)	No	UPL
KCP 5.1.2/06 (filled under KCP 10.3.1.2/01)	Knautz, T., Horstmann, C.	2023	Boscalid + Prothioconazole (350 + 200 g/kg – WG): Chronic Oral Toxicity Test on the Honey Bee (Apis mellifera L.) in the Laboratory UPL/2023/1209 ibacon GmbH 176531136 (Analytical phase, KCP 10.3.1.2/01)	No	UPL
KCP 5.1.2/07 (filled under KCP 10.3.1.1.1/02)	Chwiesko, D., Horstmann, C.,	2023	Boscalid + Prothioconazole (350 + 200 g/kg – WG): Acute Contact and Oral Toxicity to Bumblebees (Bombus terrestris L.) in the Laboratory UPL/2023/1279 176531105 ibacon GmbH (Analytical phase, KCP 10.3.1.1.1/02)	No	UPL
KCP 5.1.2/08 (filled under KCP 10.6.2/01)	Stürtz, S., Horstmann, C.,	2023a	Boscalid + Prothioconazole (350 + 200 g/kg - WG): Effects on Terrestrial (Non-Target) Plants: Vegetative Vigour Test, UPL/2023/0743 176531087 ibacon GmbH (Analytical phase, KCP 10.6.2/01)	No	UPL
KCP 5.1.2/09 (filled under KCP 10.6.2/01)	Stürtz, D., Horstmann, C.	2023b	Boscalid + Prothioconazole (350 + 200 g/kg - WG): Effects on Terrestrial (Non-Target) Plants: Seedling Emergence and Seedling Growth Test UPL/2023/0742	No	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
10.6.2/02)			ibacon GmbH 176531086 (Analytical phase, KCP 10.6.2/02)		
KCP 5.2/01	Rastogi T.	2022b	Development and Validation of Multi-Residue Method QuEChERS for Determination of Boscalid in Different Matrices of Plant Origin Study UPL/2022/0592 Eurofins Agrosience Services, EAG Laboratories GmbH S22-000980 GLP Unpublished	N	UPL
KCP 5.2/02	Gustloff C.	2022	Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Boscalid in Different Matrices of Plant Origin Study UPL/2022/0875 Eurofins Agrosience Services, EAG Laboratories GmbH S22-05632 GLP Unpublished	N	UPL
KCP 5.2/03	Rastogi T.	2023	Development and Validation of Analytical Methods for Determination of Boscalid and its Metabolite M510F01 and Bound Residues in Different Matrices of Animal Origin Study UPL/2022/2043 Eurofins Agrosience Services, EAG Laboratories GmbH S22-00981 GLP Unpublished	N	UPL
KCP 5.2/04	Spielvogel Ch., Timmann A.	2023	Amendment No. 1 to Final Report Independent Laboratory Validation of Analytical Methods for Determination of Boscalid and its Metabolite M510F01 and Bound Residues of the Metabolites M510F52 and M510F53 in Different Matrices of Animal Origin – Amended 09 April 2025 Study UPL/2023/0500 Eurofins Agrosience Services, EAG Laboratories GmbH S22-05633 (EAG-2202V) GLP Unpublished	N	UPL
KCP 5.2/0-6	Rastogi T.	2022	Independent Laboratory Validation of the Analytical method for Determination of Residues of Boscalid in Honey UPL/2022/0845	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
			Eurofins Agrosience Services EcoChem GmbH S22-00783 GLP Unpublished		
KCP 5.2/0307	Rastogi T.	2022	Validation of Analytical Method for Determination of Boscalid and its Metabolites M510F47, M510F49 and M510F64 in Ground Water and Surface Water UPL/2022/2227 Eurofins Agrosience Services EAG Laboratories GmbH S22-00979 GLP Unpublished	N	UPL
KCP 5.2/0408	Kaiser M.	2023	Independent Laboratory Validation of an Analytical Method for Determination of Boscalid and its Metabolites M510F47, M510F49 and M510F64 in Ground and Drinking Water, UPL/2022/21136, Eurofins Agrosience Services EcoChem GmbH S22-01028 GLP Unpublished	N	UPL
KCP 5.2/0509	Rastogi T.	2022	Development and Validation of an Multi-Residue Method QuEChERS for the Determination of Boscalid and its Metabolite M510F01 in Body Fluids UPL/2022/2054 Eurofins Agrosience Services, EAG Laboratories GmbH S22-00982 GLP Unpublished	N	UPL
KCP 5.1.2/10 KCP 5.2/0610	Pearson J.	2022	Prothioconazole-desthio: Method Validation in Crops Company Report No. QG/20/011 Battelle UK GLP Unpublished	N	UPL
KCP 5.1.2/11	Giancola D.	2023	Hydroxy Metabolites of prothioconazole-desthio: Method Validation in Crops Report No. QG/22/001 Battelle UK GLP Unpublished	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
KCP 5.1.2/12	Phipps B.	2024	Triazole Derived Metabolites (TDM's) (1,2,4-Triazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops Report No. QG/20/012 Battelle UK GLP Unpublished	N	UPL
KCP 5.1.2/13 (Filled in KCA 6.10/03 (CP 7.3.7))	Lebrun F.	2024	Magnitude of the residue of triazole derivative metabolites in phacelia honey in Northern and Southern Europe – 2023 Report No. 644-2023 Analytical phase code. S23-101384-L1 (TES-2307) TESTAPI GLP Unpublished	N	UPL Albaugh Sipcam Barclay
KCP 5.2/0911	Boubakri N.	2023	Independent Laboratory Validation of Multi Residue Method for Determination of Prothioconazole-desthio in Different Matrices of Plant Origin Report No. S21-08354 Eurofins Agrosience Services GLP Unpublished	N	UPL
KCP 5.2/0912	Kleinhenz D.	2023	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Food of Animal Origin Report No. S21-08355 Eurofins Agrosience Services GLP Unpublished	N	UPL
KCP 5.2/0913	Rastogi T.	2023	Independent Laboratory Validation of Prothioconazole-desthio in Different Matrices of Animal Origin Report No. S21-08868 GLP Eurofins Agrosience Services Unpublished	N	UPL
KCP 5.2/4014	Hitchens J.	2023	Prothioconazole-desthio and hydroxy metabolites: Method Validation in Honey Report No. QG/21/009 Battelle UK GLP Unpublished	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
KCP 5.2/44 15	Wanger J.	2023	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Honey Report No. S21-08357 Eurofins Agroscience Services GLP Unpublished	N	UPL
KCP 5.2/44 16	Kaiser M.	2022	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Soil Eurofins Agroscience Services Report No. S21-08358 GLP Unpublished	N	UPL
KCP 5.2/44 17	Kaiser D.	2022	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Water Eurofins Agroscience Services Report No. S21-08359 GLP Unpublished	N	UPL
KCP 5.2/44 18	Jooß S.	2023	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Water Report No. S21-08869 Eurofins Agroscience Services GLP Unpublished	N	UPL
KCP 5.2/44 19	Boubakri N.	2023	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Urine Report No. S21-08361 Eurofins Agroscience Services GLP Unpublished	N	UPL

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
CP 5.1.2	Weeren, R.D., Pelz, S.	1999	Validation of DFG method S19 for the determination of BAS 510 F in various plant materials Az. M8020/99 1999/11461 GLP Unpublished	N	BAS
CP 5.1.2	Reichert, N.	2001	Independent laboratory validation of a method of analysis for the determination of BAS 510 F in white cabbage, rape (seed), hop, and lettuce IF-100/35725-00 2000/1014886 GLP Unpublished	N	BAS
CP 5.1.2	Class, T.	2001	Assessment and validation of the adapted multi-residue method DFG S19 for the determination of BAS 510 F and its metabolite M510F01 in animal matrices Report no. P/B 453 G. 2000/1017227 GLP, Unpublished	N	BAS
CP 5.1.2	Funk, H., Mackenroth, Ch.	2001	Validation of BASF method no. 445/0: determination of BAS 510 F in plant matrices; study code 41840. 2000/1012404 GLP Unpublished	N	BAS
CP 5.1.2	Fabian, E.	2001	The validation of BASF method 476/0: the determination of BAS 510 F residues (as M510F53) in liver and milk by microwave treatment Study code 96997. 2000/1017224 GLP Unpublished	N	BAS
CP 5.1.2	Keller, W.	1998b	Validation of analytical method no. 411, determination of BAS 510 F ai residues in water; study no. 41877. 1998/10922 GLP, Unpublished	N	BAS

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
CP 5.1.2	Grote, C	2003b	Report Amendment No.1 to Validation of analytical method No. 411. Determination of BAS 510 F ai residues in water; BASF Doc ID 2003/1000976 unpublished	N	BAS
CP 5.1.2	Grote, Ch.	2001	Validation of analytical method no. 411/0, GC/MS determination of BAS 510 F ai residues in surface water; study code 110241. 2001/1008955 GLP, unpublished	N	BAS
CP 5.1.2	Grote, C	2003	Report Amendment No.1 to Validation of analytical method No. 411/0. GC/MS determination of BAS 510 F ai residues in surface water BASF Doc ID 2003/1000975 unpublished	N	BAS
CP 5.1.2	Keller, W.	1998	Validation of analytical method no. 408/1, GC MS determination of BAS 510 F active ingredient residues in soil and sediment after methanol extraction; study code 48541. 1998/11314 GLP, Unpublished	N	BAS
CP 5.1.2	Grote, C.	2003	Report Amendment No.1 to Validation of analytical method No. 408/1. GC-MS determination of BAS 510 F active ingredient residues in soil and sediment after methanol extraction; BASF Doc ID 2003/1000977 Unpublished	N	BAS
CP 5.1.2	Zangmeister, W.	2000	Validation of analytical method 460, determi nation of BAS 510 F (Reg.no. 300355) in air by GC-MS; study code 41886. BASF Doc ID 2000/1014992 GLP Unpublished	N	BAS
CP 5.1.2	Kampke-Thiel, K.	2001	Independent laboratory validation of the adapted multi-residue method DFG S19 for the determination of BAS 510 F and its metabolite M510F01 in animal matrices PTRL Europe Study No. P453G. 2000/1017226 GLP	N	BAS

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		
CP 5.1.2	Grosshans, F.	2001	The validation of BASF method 471/0: The determination of BAS 510F and the metabolite M510F01 in animal matrices Study code 42392. 2000/1017223 GLP Unpublished	N	BAS
CP 5.1.2	Bross, M.	2001	Investigations on the extractability of 14C-BAS 510 F residues from plant matrices; study code 73479. 2001/1001739 GLP Unpublished	N	BAS
CP 5.1.2	Heinemann , O.	2000a	Analytical determination of residues of JAU 6476 and dethio-JAU 6476 in/on cereals by HPLC/MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00598, Edition Number: M 028457-01-1 Method Report No.: MR 401/99 EPA MRID No.: 46477702 Date: 2000-03 20 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2	Heinemann , O.	2000b	Analytical determination of residues of JAU6476 and JAU6476 desthio in/on cereals and canola by HPLC-MS/MS (method modification 00598/M001) Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00598/M001, Edition Number: M 047681-01-1 Method Report No.: MR 689/99 EPA MRID No.: 46477703 Date: 2000-08 28 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Weeren, R. D.; Pelz, S.	2000	Modification M033 of method 00086: Validation of DFG method S 19 (extended revision) for the determination of residues of JAU 6476 desthio in materials of plant and animal origin Dr. Specht & Partner, Chemische Laboratorien GmbH, Hamburg, Germany Bayer CropScience, Report No.: 00086/M033, Edition Number: M 027637-01-1 Date: 2000-11 20	N	Bayer CropScience

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP/GEP: yes, unpublished		
CP 5.1.2 CP 5.2	Class, T.	2001	Independent laboratory validation of DFG method S19 (extended revision) for the determination of residues of JAU 6476 desthio (Bayer method 00086/M033) in plant materials PTRL Europe GmbH, Ulm, Germany Bayer CropScience, Report No.: P/B 484 G, Edition Number: M 033019-01-1 Method Report No.: P/B 484 G Date: 2001-05 15 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Heinemann, O.	2001a	Analytical determination of residues of JAU6476-3 hydroxy desthio, JAU6476-4 hydroxy desthio, and JAU6476 desthio in/on matrices of animal origin by HPLC MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00655, Edition Number: M 037709-01-1 Method Report No.: MR 537/00 EPA MRID No.: 46477704 Date: 2001-02 27 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Heinemann, O.	2001c	Analytical determination of residues of JAU6476-3 hydroxy-desthio, JAU6476-4 hydroxy desthio, and JAU6476 desthio in milk by HPLC MS/MS (00655/M001) Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00655/M001, Edition Number: M 021546-01-1 Method Report No.: MR 170/01 Date: 2001-05 04 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Dubey, L.	2001	Independent laboratory validation of Bayer methods 00655 and 00655/M001 for the determination of residues of JAU6476-3 hydroxy desthio, JAU6476-4 hydroxy desthio, and JAU6476 desthio in/on matreces of animal origin by HPLC MS/MS Battelle Geneva Research Centres, Geneva, Switzerland Bayer CropScience, Report No.: A 14-01-01, Edition Number: M 081595-01-1 Method Report No.: A-14-01 01 Date: 2001-10 16 GLP/GEP: yes,	N	Bayer CropScience

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		
CP 5.1.2	Schramel, O.	2000	Residue analytical method 00610 (MR-643/99) for the determination of JAU 6476 and the metabolites JAU6476 desthio and JAU6476-S methyl in soil by HPLC MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: MR-643/99, Edition Number: M 041798-01-1 Method Report No.: MR 643/99 EPA MRID No.: 46246543 Date: 2000-07- 13 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2	Steinhauer, S.	2001	Enforcement method 00086/M038 for the determination of the residues of JAU 6476 desthio in soil - validation of DFG method S 19 (extended revision) – Dr. Specht & Partner, Chemische Laboratorien GmbH, Hamburg, Germany Bayer CropScience, Report No.: 00086/M038, Edition Number: M 067970-01-1 Method Report No.: BAY 0107V Date: 2001-07 25 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2	Sommer, H.	1999	Method for the determination of JAU 6476 and SXX 0665 in test water from aquatic toxicity tests by HPLC [Tox/Ecotox method] Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00586, Edition Number: M 012801-01-1 Method Report No.: MR 291/99 Date: 1999-05 18 GLP/GEP: no, unpublished	N	Bayer CropScience
CP 5.1.2	Sommer, H.	2001a	Method for determination of JAU6476-S methyl in test water from aquatic toxicity tests by HPLC UV [Tox/Ecotox method] Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00699, Edition Number: M 052730-01-1 Method Report No.: MR 250/01 Date: 2001-06 21 GLP/GEP: no,	N	Bayer CropScience

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		
CP 5.1.2 CP 5.2	Sommer, H.	2001b	Enforcement method 00684 for determination of JAU 6476 and JAU 6476 desthio in drinking and surface water by HPLC MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00684, EditionNumber: M 079449-01-1 Method Report No.: MR 105/01 Date: 2001-10 23 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Maasfeld, W.	2002a	Method for the determination of JAU 6476 in air by HPLC MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00724, Edition Number: M 032554-01-1 Method Report No.: MR 601/01 Date: 2002-01 22 GLP/GEP: yes, unpublished	N	Bayer CropScience
CP 5.1.2 CP 5.2	Maasfeld, W.	2002b	Method for the determination of JAU 6476 desthio (SXX 0665) in air by HPLC-MS/MS Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 00731, Edition Number: M 036729-01-1 Method Report No.: MR 003/02 Date: 2002-02 13 GLP/GEP: yes, unpublished	N	Bayer CropScience

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 Boscalid

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

A 2.1.1.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.1)

A 2.1.1.1.1 Analytical method 1

A 2.1.1.1.1.1 Method validation

Comments of zRMS:	The analytical method was successfully validated and is considered suitable for the determination of residues of boscalid in crops: 1. Wheat grains (Dry commodities) 2. Wheat straw (Dry commodities) 3. Lettuce (High water content commodities) 4. Whole orange (High acid content commodities) 5. Oilseed rape seeds (High oil content commodities) with an LOQ of 0.01 mg/kg. Mean recoveries were in the range of 60 – 120% with relative standard deviations of $\leq 30\%$ for all matrices at each level (0.01 and 0.1 mg/kg). The method is acceptable.
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Reference: KCP 5.1.2/01

Report Validation of the Analytical Method for Determination of Boscalid in Different Matrices of Plant Origin, Rastogi, T., 2022, Study Number: S22-06799, Sponsor Study Number: UPL/2022/2099

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

After preparation of the sample material for analysis (the sample material was thoroughly homogenised in a knife mill using dry ice), wheat grain, lettuce, whole orange and oilseed rape seed matrices were stored at $\leq -18^{\circ}\text{C}$ (frozen condition) in the dark whereas wheat straw matrix was stored at room temperature in the dark before weighing, fortification and extraction.

Samples of investigated matrices were extracted with the mixture of methanol/water/2N HCl (70/25/5, v/v/v). The samples were shaken using horizontal shaker. An aliquot of the raw extract was then transferred into the centrifuge tubes and centrifuged for about 5 minutes. The samples were diluted (DF2) in methanol/water (1/1, v/v) prior to the LC-MS/MS analysis for the determination of boscalid.

Chromatographic and Mass Spectrometric Conditions

HPLC-MS/MS determination, MRM scan, positive ion mode with monitorization of 343→370 ion for quantification and 343→140 for confirmation (343→271 used as confirmation mass transition only for Oilseed rape matrix)

HPLC systemt: 1290 Infinity Binary LC System, Agilent Technologies

Pre-column: Phenomenex C18 4x3mm

Column: Thermo Betasil C18 column: Length: 100 mm, i.d.: 2.1 mm, particle size: 5 μm .

Column oven temperature: 40°C

Injection Volume: 30 µL

Mobile Phase:

Eluent A: Water containing 0.1% (v/v) formic acid

Eluent B: Methanol containing 0.1%(v/v) formic acid

Retention Time: 3.0 minutes (approximately)

Results and discussions

The objective of the study was to develop and validate the analytical method for the determination of boscalid in/on different matrices of plant origin in accordance to guidance document SANTE/2020/12830, rev.1 for risk assessment.

The limit of quantification was 0.01 mg/kg for all matrices.

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

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall Mean Recovery (%)	Overall Rel.Std. Dev. (%)	Comments
MassTransition:343m/z→307m/z(Quantification)							
Wheat grains	Boscalid	0.01	104	2.7	102	3.9	Range: 99.6 - 107
		0.1	99.8	4.3			Range: 95.6 - 105
Wheat straw	Boscalid	0.01	88.5	5.1	87.5	3.7	Range: 80.8 - 92.8
		0.1	86.6	1.0			Range: 85.4 - 87.6
Lettuce	Boscalid	0.01	106	4.3	101	9.8	Range: 102 - 114
		0.1	94.9	11			Range: 85.6 - 107
Whole orange	Boscalid	0.01	105	2.0	98.7	7.0	Range: 102 - 108
		0.1	92.5	3.3			Range: 88.0 - 95.6
Oilseed rape seed	Boscalid	0.01	94.7	6.6	92.1	5.8	Range: 86.4 - 101
		0.1	89.5	3.3			Range: 85.6 - 93.8
MassTransition:343m/z→140m/z(Confirmation) OnlyforOilseedrapeseedmatrix:MassTransition:343m/z→271m/z(Confirmation)							
Wheat grains	Boscalid	0.01	103	2.2	102	3.4	Range: 101- 107
		0.1	101	4.2			Range: 96.8 - 107
Wheat straw	Boscalid	0.01	88.3	7.1	87.3	5.2	Range: 80.8 - 95.2
		0.1	86.2	2.2			Range: 83.2 - 88.8
Lettuce	Boscalid	0.01	99.2	11	97.3	7.4	Range: 97.2 - 103
		0.1	95.3	11			Range: 86.4- 107
Whole orange	Boscalid	0.01	105	2.0	98.0	7.7	Range: 99.6 - 108
		0.1	91.4	2.5			Range: 87.6 - 93.6
Oilseed rape seed	Boscalid	0.01	96.0	6.9	93.2	6.2	Range: 86.4 - 103
		0.1	90.4	3.6			Range: 86.4 - 95.4

Table A 2: Characteristics for the analytical method used for validation of boscalid residues in plants

	Boscalid
Specificity	Demonstrated by validation of two (2) mass transitions. Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below what would correspond to an analyte level of 30% of the LOQ.
Calibration (type, number of data points)	Linear regression with 1/x weighting.

	Boscalid
Specificity	Demonstrated by validation of two (2) mass transitions. Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below what would correspond to an analyte level of 30% of the LOQ.
	Regression residuals randomly distributed Correlation coefficients (R) ≥ 0.99 Wheat Grain (Linear Regression (1/x weighting): Boscalid (343/307)) $y=2.43e+005x+4.9e+003(r=0.9997)$ Boscalid (343/140)) $y=4.95e+004x+1.51e+003(r=0.9996)$ Wheat straw (Linear Regression (1/x weighting): Boscalid (343/307): $y=2.89e+005x+808(r=0.9998)$ Boscalid (343/140): $y=5.77e+004x+324(r=0.9999)$ Lettuce (Linear Regression (1/x weighting): Boscalid (343/307): $y=3.98e+005x+4e+004(r=0.9992)$ Boscalid (343/140): $y=8.1e+004x+8.71e+003(r=0.9992)$ Whole Orange (Linear Regression (1/x weighting): Boscalid (343/307): $y=2.84e+005x+4e+003(r=0.9993)$ Boscalid (343/140): $y=5.88e+004x+634(r=0.9993)$ OSR seeds (Linear Regression (1/x weighting): Boscalid (343/307): $y=2.87e+005x+2.13e+004(r=0.9992)$ Boscalid (343/271) : $y=9.49e+004x+2.98e+003(r=0.9989)$
Calibration range	Matrix-matched calibration standards. A minimum of seven (7) concentration levels. Single determination. Injection of standard solutions spread over the whole acquisition batch. Concentration range: 0.075 ng/mL to 7.5 ng/mL. Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg. Coverage: 30% of the LOQ to at least + 20% of the highest analyte concentration level detected in a diluted sample extract. The validated range does not exceed two (2) orders of magnitude.
Assessment of matrix effects is presented	Yes Insignificant (< 20%) for all matrices except wheat grain and wheat straw. Matrix-matched standards were used for quantification throughout the study.
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg
Stability of Working Solution	The stability of the working solution (i.e. stock, fortification and intermediate calibration solutions) of boscalid in methanol was demonstrated for 16 days when stored at 1°C to 10°C in the dark in the study S22-00980.
Stability of Final Extracts	Recoveries within 70% - 120% in all matrix extracts for at least 11 days when stored at typically 1 °C to 10 °C in the dark.

Conclusion

The method was successfully validated for the determination of boscalid in/on different matrices of plant origin (dry, high water, high acid and high oil commodities) from the tested LOQ of 0.01 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and meets the requirements of SANTE/2020/12830, rev. 2.

A 2.1.1.1.1.2 Confirmatory method

No confirmatory method is required (two mass transitions).

A 2.1.1.1.1.3 Extraction efficiency

Extraction efficiency of the solvent systems as used in the data generation method S22-06799 is demonstrated in the below study.

Comments of zRMS:	The extraction efficiency is considered sufficiently proven for the multi-residue method (Rastogi, T., 2022, Study Code: S22-00980) and the data generation / risk assessment method (Rastogi, T., 2022, Study Code: S22-06799) compared to the metabolism method (DRAR for boscalid, 2018).The suitability of the extraction procedures from the multi-residue method and the data generation / risk assessment method in wheat grain, wheat whole plant and oilseed rape seeds is demonstrated according to the criteria from the guideline SANTE/2017/10632. rev.4.
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Reference: KCP 5.1.2/02

Report Cross-Validation - Comparing Amounts of Boscalid extracted from Samples of Plant Origin with incurred Residues using three different Solvent Systems, Rastogi, T., 2023, Study Number: S22-00983, Sponsor Study Number: UPL/2023/1062

Guideline(s): Yes, SANTE/2017/10632, rev. 4 and SANTE/2020/12830, Rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Study Objective

The objective of the study was to compare residue amounts of boscalid extracted from samples of wheat whole plant (high water content matrix), wheat grains (dry matrix) and oilseed rape seeds (high oil content matrix) with incurred residues when extracting with different solvent systems as used in pre and post registration methods and when extracting with different solvent systems as were used in metabolism studies in accordance to the technical guideline on the evaluation of extraction efficiency of residue analytical methods, SANTE/2017/10632, rev. 4. All methods were validated in accordance with SANTE/2020/12830, rev. 2.

Materials and methods

The samples analysed in the current study were derived from the raw agricultural commodity of matrices obtained in the corresponding residues studies/analytical phases (wheat whole plant, wheat grains and oilseed rape seeds). Samples from those matrices were used for the extraction with the different solvent systems.

The samples were homogenised with the respective analytical phases.

Table A 3: Extraction methods used for the cross validation

Method 1: Multi-residue method	
Reference(s)	S22-00980
Solvent System for Extraction	Addition of water (if necessary) and extraction with acetonitrile
Validation Status	All matrix categories occurring in this study have previously been validated at the test facility as can be taken from the reference given above
Method 2: Data generation / risk assessment method	
Reference(s)	S22-06799 [KCP 5.1.2/01]
Solvent System for Extraction	Mixture of methanol, water and hydrochloric acid
Validation Status	All matrix categories occurring in this study have previously been validated at the test facility as can be taken from the reference given above.
Method 3: Metabolism method	
Reference(s)	Metabolism study [Vol 3-B7, RAR 2018]
Solvent System for Extraction	Extraction with methanol
Validation Status	A full validation was part of this study

Results and discussions

Table A 4: Summary evaluation of extraction efficiency of residue analytical methods

Item	Activity, Result, Assessment															
Analyte	Boscalid															
Matrices	Wheat grain (dry commodity), wheat whole plant (high water content commodity), oilseed rape seeds (high oil content matrix)															
Method References	Methods developed in Study S22-00980 and S22-06799 [KCP 5.1.2/01], Metabolism Method [Vol 3-B7, RAR 2018]															
LOQ	0.01 mg/kg (lowest validated fortification level) for all matrices															
LOD	≤30 % of the LOQ															
Principle of the Analytical Procedure	Homogenisation: Dry ice, knife mill or cutter Method S22-00980 (Method 1): Extraction: Acetonitrile and addition of water (if needed). Ratio for Whole Plant: 1 mL of extraction solvent per g of matrix Ratio for Grain: 2 mL of extraction solvent per g of matrix Ratio for Oilseed rape seed: 4 mL of extraction solvent per g of matrix Clean-up: Dispersive SPE with primary/secondary amine (PSA) Liquid/liquid partition: Addition of salt mixture Dilution with acetonitrile/water (1/1, v/v) Sample concentration in final extract: 0.05 g sample per mL of extract Method S22-06799 (Method 2): Extraction: Methanol/water/hydrochloric acid (70/25/5 v/v). Ratio: 20 mL of extraction solvent per g of matrix Dilution with methanol/water (1/1 v/v) Sample concentration in final extract: 0.025 g sample per mL of extract Metabolism Method (Method 3): Extraction: Methanol Ratio: 20 mL of extraction solvent per g of matrix Dilution with water Sample concentration in final extract: 0.025 g sample per mL of extract															
Selectivity and Specificity	Demonstrated by evaluation of one (1) mass transitions for Method 1 and Method 2 and by validation of two (2) mass transitions for Method 3. Analyte levels or chromatographic interferences of unknown compounds in control sample extracts were in some cases above of what would correspond to an analyte level of 30% of the LOQ.															
Matrix Effects on Analyte Detection	For Method 1 Method 2: Matrix effects have been evaluated during the validation studies. For the Metabolism Method (evaluated during this study): Significant (≥ 20%) for wheat whole plant. Insignificant (< 20%) for wheat grain and oilseed rape seeds.															
Calibration	Matrix matched calibration standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Method 1: 0.15 ng/mL to 10 ng/mL corresponding to 0.003 mg/kg to 0.20 mg/kg Method 2 and Method 3: 0.075 ng/mL to 7.5 ng/mL corresponding to 0.003 mg/kg to 0.30 mg/kg Coverage: 30% of the LOQ to at least + 20% of the highest analyte concentration level detected in a diluted sample extract. The validated range does not exceed two (2) orders of magnitude.															
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed. Correlation coefficients (R) ≥ 0.99															
Accuracy and Precision	The following fortification experiments were conducted: Method 1 and Method 2: One fortification at LOQ, one fortification at 10x LOQ and one fortification at 1000x LOQ Method 3: Five fortifications at LOQ, five fortifications at 10x LOQ and one fortification at 1000x LOQ Mean recoveries for the evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2: <table><tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr><tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr><tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr><tr><td>> 0.1 - ≤ 1.0</td><td>70 - 110</td><td>≤ 15</td></tr><tr><td>> 1</td><td>70 - 110</td><td>< 10</td></tr></table>	Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)	≤ 0.01	60 - 120	≤ 30	> 0.01 - ≤ 0.1	70 - 120	≤ 20	> 0.1 - ≤ 1.0	70 - 110	≤ 15	> 1	70 - 110	< 10
Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)														
≤ 0.01	60 - 120	≤ 30														
> 0.01 - ≤ 0.1	70 - 120	≤ 20														
> 0.1 - ≤ 1.0	70 - 110	≤ 15														
> 1	70 - 110	< 10														

Stability of Analyte in Standard Solutions	Stability of analyte(s) in standard solutions has been demonstrated in Analytical Phase S22-01575-L1 [please refer to dRR part B7, KCP 7.2.3.3/01] for 177 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark.
Extraction Efficiency	Two samples with incurred residues were analysed (three replicates) following the methods described with evaluation of one (1) mass transition. The results were compared to the results obtained using the extraction solvent systems from the metabolism study [Vol 3-B7, RAR 2018].

Conclusion

The method based on the metabolism method (Method 3) was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for the determination of boscalid with an LOQ of 0.01 mg/kg for wheat grain and wheat whole plant and oilseed rape seeds. The extraction efficiency is considered sufficiently proven for the Multi-residue method (Method 1 [S22-00980]) and the data generation / risk assessment method (Method 2 [S22-06799]) compared to the metabolism method (Method 3) [Vol 3-B7, RAR 2018]. The following table shows the average extraction efficiency of each method (Method 1 and Method 2) and for each matrix when compared to the metabolism method (Method 3).

	Wheat (Whole Plant)	Wheat (Grain)	OSR (Seeds)
Method 1	89.8%	110%	88.0%
Method 2	91.1%	94.3%	101%

Therefore, the suitability of the extraction procedures from the Multi-residue method (Method 1 [S22-00980]) and the data generation / risk assessment method (Method 2 [S22-06799]) in wheat grain, wheat whole plant and oilseed rape seeds is demonstrated according to the criteria from the guideline SANTE/2017/10632. Rev.4.

A 2.1.1.2 Description of Methods for the Analysis of Animal Matrices (KCP 5.1)

A 2.1.1.2.1 Analytical method 1

A 2.1.1.2.1.1 Method validation

Comments of zRMS:	Please refer to point A 2.1.2.2.2.1 (reference KCP 5.2/05).
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Reference:	KCP 5.1.2/ 03 (KCP 5.2/0-5)
Report	Validation of the Analytical Method for the Determination of Residues of Boscalid in Honey, Report Amendment 1, Sahvorost, N., 2022, Study No. S22-00776, Sponsor Study No. UPL/2022/0354.
Guideline(s):	Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

The study by Sahvorost, N., 2022, Study No. S22-00776 is summarized in point in point A 2.1.1.2.1.1 A 2.1.2.2.2.1 (reference KCP 5.2/05). The analytical method is identical that the one used for monitoring purposes and was also used to generate pre-authorization data (please refer to dRR Part B7, point A2.1.7.1, KCP 6.10/01, CP 7.2.7).

A 2.1.1.3 Description of Methods for the Analysis of Water (KCP 5.1)

A 2.1.1.3.1 Analytical method 1

A 2.1.1.3.1.1 Method validation

Comments of zRMS:	The analytical method was successfully validated according to the SANTE/2020/12830, Rev.2 for the determination of the boscalid and prothioconazole in reconstituted water and in 1X AAP medium. The LOQ was 250.306 µg/L (boscalid) and 139.454 µg/L (prothioconazole) in reconstituted water and 196.669 µg/L (boscalid) and 109.571 µg/L (prothioconazole) in 1X AAP medium.
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Reference:	KCP 5.1.2/04
Report	Validation of Analytical Method for Determination of Active Ingredient Concentration and Stability of Boscalid and Prothioconazole in Matrix, following the Application of Boscalid 350 g/kg and Prothioconazole 200 g/kg WG, Mahera N., 2023, JRF Study Number: 228-2-13-33021, Sponsor Study Number: UPL/2022/21191.
Guideline(s):	Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

This study was performed to validate the analytical method for determination of boscalid and prothioconazole concentration in matrix (reconstituted water, and 1X AAP), and to establish the stability of Boscalid 350 g/kg + Prothioconazole 200 g/kg WG in matrix (Reconstituted water and 1×AAP media) for 48 h, 72 h and 96 h by LC-MS/MS.

The test item working solutions were prepared in reconstituted water and in 1X AAP. All the final working solutions prepared for the experiments matrix effect, specificity, linearity, limit of quantification (LOQ), recovery and repeatability (%RSD), stability (standard solution stability and test item stability), were injected onto HPLC instrument for analysis. The analytical method parameters are provided below:

Instrument:	LC-MS/MS [Shimadzu, LCMS-8050 coupled with Nexera X2 system]
Column:	BDS Hypersil C18, [100 mm x 4.6 mm (i.d.), 5 µm]
Injection Volume:	2 µL
Flow Rate:	0.5 mL/minute
Cooler Temperature:	10°C
Column Temperature:	25°C
Mobile Phase A:	Methanol (85% v/v)
Mobile Phase B:	5 mM Ammonium Formate in Milli-Q-Water containing 0.1 % Formic acid (15% v/v)
Retention Time:	Boscalid- 2.705 minute Prothioconazole- 3.485 minutes
MS Parameters, MRM(Q1/Q3):	Boscalid: 342.95/271.20 Prothioconazole: 343.85/189.10; 343.85/154.25

Extraction procedure: A volume of 0.050 mL of matrix samples were transferred to pre-labelled autosampler vial containing 0.950 mL of Diluent [Methanol: Water (90:10 %v/v)]. Vortexed to mix.

Results and discussions

The analytical method for the determination of Boscalid and Prothioconazole in matrix, following the application of Boscalid 350 g/kg and Prothioconazole 200 g/kg WG was successfully validated with a limit of quantification (LOQ) of 250.306 µg/L (Boscalid) and 139.454 µg/L (Prothioconazole) in reconstituted water and 196.669µg/L (Boscalid) and 109.571µg/L (Prothioconazole) in 1X AAP medium. No additional confirmatory method or an ILV is required.

Table A 5: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
1X AAP	Boscalid	LOQ Level	96.16	0.96	Range: 94.70-97.01
		High Dose Level	95.42	0.42	Range: 95.08-96.10
	Prothioconazole	LOQ Level	100.28	2.79	Range: 95.86-102.42
		High Dose Level	101.63	0.54	Range: 100.99-101.66
Reconstituted Water	Boscalid	LOQ Level	97.87	1.07	Range: 97.10-99.67
		High Dose Level	94.94	4.95	Range: 86.56-97.33
	Prothioconazole	LOQ Level	99.32	2.22	Range: 97.38-102.98
		High Dose Level	102.75	4.83	Range: 93.89-105.32

Table A 6: Characteristics for the analytical method used for validation of boscalid residues in reconstituted water and 1X AAP

	Boscalid (in 1X AAP)	Boscalid (in reconstituted water)
Specificity	Blank value < 30 % LOQ No interference	Blank value < 30 % LOQ No interference
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points r= 0.9999 Regression residuals presented in residual plot with random distribution	Individual calibration data presented Calibration line equation presented 7 data points r= 0.9999 Regression residuals presented in residual plot with random distribution
Calibration range (µg/L)	50.175 to 5017.513	50.175 to 5017.513
Assessment of matrix effects is presented	Yes	Yes
Limit of determination/quantification (µg/L)	LOD: 50.175 LOQ: 196.669	LOD: 50.175 LOQ: 250.306

Table A 7: Characteristics for the analytical method used for validation of prothioconazole residues in reconstituted water and 1X AAP

	Prothioconazole (in 1X AAP)	Prothioconazole (in reconstituted water)
Specificity	Blank value < 30 % LOQ No interference	Blank value < 30 % LOQ No interference
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points r= 0.9999 Regression residuals presented in residual plot with random distribution	Individual calibration data presented Calibration line equation presented 7 data points r= 0.9999 Regression residuals presented in residual plot with random distribution
Calibration range (µg/L)	30.290 to 3029.037	30.290 to 3029.037
Assessment of matrix effects is presented	Yes	Yes
Limit of determination/quantification (µg/L)	LOD: 30.290 LOQ: 109.571	LOD: 30.290 LOQ: 139.454

Conclusion

From the results of the validation, it is concluded that the method is sensitive, precise, and accurate for the analysis of the active ingredients concentration and stability of Boscalid 300 g/Kg and Prothioconazole

200 g/kg WG in the media, viz., reconstituted water for the daphnia study, and 1X AAP media for the alga study. The stability of test item was established up to 96 h for 1×AAP media and up to 48 h for reconstituted water. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.1.2 Confirmatory method (if required)

No confirmatory method is required.

A 2.1.1.3.2 Analytical method 2

A 2.1.1.3.2.1 Method validation

Comments of zRMS:	The analytical method was validated to verify the concentration of boscalid and prothioconazole in water solutions with LOQ of 0.1 g test item/L, corresponding to 35.5 mg boscalid/L and 20.4 mg prothioconazole/L. All validity criteria for the analytical method have been met according to the SANTE/2020/12830, Rev.2 for risk assessment purpose.
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Reference:	KCP 5.1.2/05
Report	Effects of Boscalid + Prothioconazole (350 + 200 g/kg – WG) on honeybees (<i>Apis mellifera</i> L.) 22-day larval toxicity test with repeated exposure, Venturi, S., 2024, Study No. BT073/23, Sponsor Study No.: UPL/2023/1235.
Guideline(s):	Yes, SANTE/2020/12830, Rev.2 (February 14, 2023)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of the analytical part of this study was to verify the concentration of active substances Boscalid and Prothioconazole in the test item water solutions at lowest and highest concentration prepared on D3 and in water used for the control.

Reagents:

Acetonitrile LC-MS Chromasolv ≥99.9% Honeywell
Water for UHPLC-MS Carlo Erba (water)
Trifluoroacetic acid for spectroscopy Merck
Test item
Analytical standards
Diluent: 50% water + 50% acetonitrile

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 100 Å- 2.6 µm – 3 x 50 mm
Pipettes:	Gilson
Analytical balance:	Mettler Toledo XP105DR
Filter NY 0.2 µm	
Laboratory glassware and equipment	

Experimental conditions

Eluent A	Water acidified with 0.1% Trifluoroacetic acid
Eluent B	Acetonitrile
Eluent ratio A/B	50/50
Flow	0.400 mL/min
Injected volume	2.0 µL
Column temperature	25°C
Run time	5.0 minutes
Analysed wavelength	250 nm
Retention time	Boscalid approx. 2.2 minutes – Prothioconazole approx. 2.9 minutes

Standard solutions

10.34 mg of Prothioconazole analytical standard were weighed and diluted into 10 mL volumetric flask with acetonitrile to obtain the SS1prot solution at 1016.3186 mg/L. 10.27 mg of Boscalid analytical standard were weighed and diluted into 10 mL volumetric flask with acetonitrile to obtain the SS1bosc solution at 1020.8380 mg/L.

Results and discussions

The HPLC-DAD analytical method for the determination of Boscalid and Prothioconazole in aqueous matrix solution treated with the test item was validated according to the guideline SANTE/2020/12830 Rev.2. The validation results are hereunder summarised.

Table A 8: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Water solution	Boscalid	LOQ Level (Nominal = 35.5 mg a.s./L)	90.03	1.52	Range: 88.47 - 91.52
		REC HIGH level (Nominal = 7100.0 mg a.s./L)	100.45	0.55	Range: 99.84 - 100.90
		Control	Boscalid not detectable		2 replicates
Water solution	Prothioconazole	LOQ level (Nominal = 20.4 mg a.s./L)	88.53	1.31	Range: 86.97 - 89.82
		REC HIGH level (Nominal = 4080.0 mg a.s./L)	98.19	0.99	Range: 96.94 - 99.11
		Control	Prothioconazole not detectable		2 replicates

Table A 9: Characteristics for the analytical method used for validation of boscalid and prothioconazole residues in water

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9997 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r= 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend
Calibration range	5.3594 to 214.3760 mg Boscalid/L (corresponding to 10.7188 mg/L – 8575.0392 mg/L of Boscalid in matrix undiluted samples)	3.0490 to 121.9582 mg Prothioconazole/L (corresponding to 6.0979 mg/L – 4878.3293 mg/L of Prothioconazole in matrix undiluted samples)

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
Assessment of matrix effects is presented	Yes	Yes
Limit of determination/quantification	0.1 g test item/L (corresponding to 35.5 mg boscalid/L)	0.1 g test item/L (corresponding to 20.4 mg prothioconazole/L)
Final standard solutions and extracts stability	The standard solutions were analysed within 24 hours from the preparation, therefore no standard solutions or extract stability were needed.	

Conclusion

All validity criteria for the analytical method have been met. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.2 Confirmatory method (if required)

No confirmatory method is required.

A 2.1.1.3.3 Analytical method 3

A 2.1.1.3.3.1 Method validation

Comments of zRMS:	The analytical method was validated to verify the concentration of boscalid and prothioconazole in feeding solution with LOQ of 1.1 g test item/L, corresponding to 9.68 mg Boscalid/L and 5.59 mg Prothioconazole/L after dilution by factor 40. All validity criteria for the analytical method have been met according to the SANTE/2020/12830, Rev.2 for risk assessment purpose.
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Reference: KCP 5.1.2/06

Report Boscalid + Prothioconazole (350 + 200 g/kg – WG): Chronic Oral Toxicity Test on the Honey Bee (*Apis mellifera* L.) in the Laboratory, Knautz. T., Horstmann, C., 2023, Study No. 176531136, Sponsor Study No.: UPL/2023/1209.

Guideline(s): Yes, SANTE/2020/12830, Rev.2 (February 14, 2023)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Samples were taken from the biological ibacon study 176531136. The concentrations of the active ingredients (Boscalid and Prothioconazole) were determined in one sample of each duplicate. The samples were stored deep frozen ($\leq -20^{\circ}\text{C}$) until analysis was performed. Analysis was performed by HPLC-UV method.

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:

The test item was dissolved in matrix (50% w/v sucrose solution, sonicated for 15 minutes and stirred for 30 minutes) to get fortified samples of about 22 g test item/L. Aliquots of these solutions were diluted in matrix to obtain fortified samples at approximately 1.1 g test item/L. Fortified samples were diluted with

solvent mixture to match calibration range.

Replicates:

Five independent replicates per fortification level. Two independent replicates of matrix blanks.

HPLC-Conditions:

System: Waters Acquity
Software: Empower 3
Column: Kinetex C18 (50 x 2.1 mm; 2.6 µm)
Mobile Phase: 45 % acetonitrile 55 % HPLC-water + 0.1 % formic acid
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: Boscalid: 1.13 minutes
Prothioconazole: 1.46 minutes

Results and discussions

The analytical method for the determination of boscalid and prothioconazole in Feeding Solution (DAA9), was successfully validated with a limit of quantification (LOQ) of 1.1 g test item/L (corresponding to 9.68 mg Boscalid/L after dilution by factor 40 and 5.59 mg Prothioconazole/L after dilution by factor 40). No additional confirmatory method or an ILV is required.

Table A 10: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Feeding solution	Boscalid	LOQ level (1.1 g test item/L)	100	1	Range: 99 - 103
		HIGH level (22 g test item/L)	97	9	Range: 83 - 106
		Control	Boscalid not detectable		2 replicates
Feeding solution	Prothioconazole	LOQ level (1.1 g test item/L)	99	1	Range: 97 - 100
		HIGH level (22 g test item/L)	95	9	Range: 81 - 103
		Control	Prothioconazole not detectable		2 replicates

Table A 11: Characteristics for the analytical method used for validation of boscalid and prothioconazole residues in feeding solution

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r= 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend
Calibration range	1 to 25 mg Boscalid/L (corresponding to 10 % of LOQ to 129 % of higher fortification level)	1 to 25 mg Prothioconazole/L (corresponding to 18 % of LOQ to 224 % of higher fortification level)
Assessment of matrix effects is presented	Yes, No significant matrix effect (≥ 20 %) was observed.	Yes, No significant matrix effect (≥ 20 %) was observed.
Limit of quantification	1.1 g test item/L	1.1 g test item/L

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
	(corresponding to 9.68 mg Boscalid/L after dilution by factor 40)	(corresponding to 40 5.59 mg Prothioconazole/L after dilution by factor 40)
Final standard solutions and extracts stability	The stock solutions of the reference items were prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed.	

Conclusion

All validity criteria for the analytical method have been met. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.3.2 Confirmatory method (if required)

No confirmatory method is required.

A 2.1.1.3.4 Analytical method 4

A 2.1.1.3.4.1 Method validation

Comments of zRMS:	The analytical method was validated to verify the concentration of boscalid and prothioconazole in test solution (contact and oral) with LOQ for contact test of 7.5 g test item/L (corresponding to 13.2 mg boscalid/L and 7.6 mg prothioconazole/L after dilution by factor 200) and for oral test of 1 g test item/L (corresponding to 7.0 mg Boscalid/L and to 4.1 mg Prothioconazole/L after dilution by factor 50). All validity criteria for the analytical method have been met according to the SANTE/2020/12830, Rev.2 for risk assessment purpose.
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Reference: KCP 5.1.2/07

Report Boscalid + Prothioconazole (350 + 200 g/kg – WG): Acute Contact and Oral Toxicity to Bumblebees (*Bombus terrestris* L.) in the Laboratory, Chwiesko. D., Horstmann, C., 2023, Study No. 176531105, Sponsor Study No.: UPL/2023/1279.

Guideline(s): Yes, SANTE/2020/12830, Rev.2 (February 14, 2023)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Samples were taken from the biological ibacon study 176531105. The concentrations of the active ingredients (Boscalid and Prothioconazole) were determined in one sample of each duplicate. The samples were stored deep frozen ($\leq -20^{\circ}\text{C}$) until analysis was performed. Analysis was performed by HPLC-UV method.

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. The samples were analysed on the day of processing.

Fortification Procedure:

- Contact Test:

The test item was dissolved in contact matrix (sonicated for 15 minutes and stirred for 30 minutes) to obtain fortification stock solutions of 150 g test item/L.

Appropriate amounts of these stock solutions were diluted in contact matrix to obtain fortified samples at 7.5 g test item/L.

- Oral Test:

The test item was dissolved in oral matrix (sonicated for 15 minutes and stirred for 30 minutes) to obtain fortification stock solutions of 25 g test item/L.

Appropriate amounts of these stock solutions were diluted in oral matrix to obtain fortified samples at 1 g test item/L.

Fortified samples were diluted with solvent mixture to match calibration range.

Replicates:

Five independent replicates per fortification level Two independent replicates of matrix blanks per matrix and measurement.

HPLC-Conditions:

System: Waters Acquity
Software: Empower 3
Column: Kinetex C18 (50 x 2.1 mm; 2.6 µm)
Mobile Phase: 45 % acetonitrile 55 % HPLC-water + 0.1 % formic acid
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: Boscalid: 1.74 minutes
Prothioconazole: 2.4 minutes

Results and discussions

The analytical method for the determination of boscalid and prothioconazole in test Solution (contact and oral), was successfully validated with a limit of quantification (LOQ) for contact test of 7.5 g test item/L (corresponding to 13.2 mg Boscalid/L and 7.6 mg Prothioconazole/L after dilution by factor 200) and for oral test of 1 g test item/L (corresponding to 7.0 mg Boscalid/L and to 4.1 mg Prothioconazole/L after dilution by factor 50). No additional confirmatory method or an ILV is required.

Table A 12: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Contact test	Boscalid	LOQ level (7,5 g test item/L)	107	3	Range: 102 - 111
		HIGH level (150 g test item/L)	108	1	Range: 106 - 109
		Control	Boscalid not detectable		2 replicates
Contact test	Prothioconazole	LOQ level (7.5 g test item/L)	106	3	Range: 101 - 110
		HIGH level (150 g test item/L)	107	1	Range: 106 - 108
		Control	Prothioconazole not detectable		2 replicates
Oral test	Boscalid	LOQ level (1 g test item/L)	96	2	Range: 95 - 99
		HIGH level (25 g test item/L)	96	1	Range: 95 - 97
		Control	Boscalid not detectable		2 replicates
Oral test	Prothioconazole	LOQ level (1 g test item/L)	99	2	Range: 97 - 100
		HIGH level (25 g test item/L)	96	1	Range: 95 - 97

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
		Control	Prothioconazole not detectable		2 replicates

Table A 13: Characteristics for the analytical method used for validation of boscalid and prothioconazole residues in contact and oral test solutions

		Boscalid	Prothioconazole
Specificity		Blank value < 30% LOQ	Blank value < 30% LOQ
Calibration (type, number of data points)	Contact Test	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9998 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend
	Oral Test	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9992 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9994 Regression residuals presented in residual plot with random distribution and no visible trend
Calibration range	Contact Test	2 to 35 mg Boscalid/L corresponding to 15 % of LOQ to 133 % of higher fortification level	2 to 35 mg Prothioconazole/L corresponding to 26 % of LOQ to 230 % of higher fortification level
	Oral Test	2 to 25 mg Boscalid/L corresponding to 14 % of LOQ to 142 % of higher fortification level	2 to 25 mg Prothioconazole/L corresponding to 25 % of LOQ to 246 % of higher fortification level
Assessment of matrix effects is presented		Yes, No significant matrix effect (≥ 20 %) was observed.	Yes, No significant matrix effect (≥ 20 %) was observed.
Limit of quantification	Contact Test	7.5 g test item/L (corresponding to 13.2 mg Boscalid/L after dilution by factor 200)	7.5 g test item/L (corresponding to 7.6 mg Prothioconazole/L after dilution by factor 200)
	Oral Test	1 g test item/L (corresponding to 7.0 mg Boscalid/L after dilution by factor 50)	1 g test item/L (corresponding to 4.1 mg Prothioconazole/L after dilution by factor 50)
Final standard solutions and extracts stability		The stock solutions of the reference items were prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed.	

Conclusion

All validity criteria for the analytical method have been met. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.4.2 Confirmatory method (if required)

No confirmatory method is required.

A 2.1.1.3.5 Analytical method 5

A 2.1.1.3.5.1 Method validation

Comments of zRMS:	The analytical method was validated to verify the concentration of boscalid and
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	prothioconazole in stock solution with LOQ 12 g test item/L corresponding to 16.9 mg boscalid/L and 9.8 mg prothioconazole/L (diluted by factor 250). All validity criteria for the analytical method have been met according to the SANTE/2020/12830, Rev.2 for risk assessment purpose.
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Reference:	KCP 5.1.2/08
Report	Boscalid + Prothioconazole (350 + 200 g/kg - WG): Effects on Terrestrial (Non-Target) Plants: Vegetative Vigour Test, Stürtz. S., Horstmann, C., 2023a, Study No. 176531087, Sponsor Study No.: UPL/2023/0743.
Guideline(s):	Yes, SANTE/2020/12830, Rev.2 (February 14, 2023)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Duplicate samples of approximately 10 mL from the homogenous stock solution and two samples of the matrix (deionised water) as control solution were taken from the biological study 176531087. The concentrations of the active ingredients Boscalid and Prothioconazole were determined in one sample of each duplicate. The samples were stored deep frozen (≤ -20 °C) until analysis was performed. Analysis was performed by HPLC-UV method.

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. The samples were analysed on the day of processing.

Fortification Procedure:

The test item was dissolved in pure water (sonicated for 5 minutes and stirred for 30 minutes) to get fortified samples of about 12 and 24 g test item /L. Fortified samples were diluted with solvent mixture to match calibration range.

Replicates:

Five independent replicates per fortification level Two independent replicates of matrix blanks.

HPLC-Conditions:

System:	Waters Acquity
Software:	Empower 3
Column:	Kinetex C18 (50 x 2.1 mm; 2.6 μ m)
Mobile Phase:	45 % acetonitrile 55 % HPLC-water + 0.1 % formic acid
Flow Rate:	0.4 mL/min
Temperature:	25°C
Injection Volume:	2 μ L
Detector:	DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm
Retention Time:	Boscalid: 1.73 minutes Prothioconazole: 2.39 minutes

Results and discussions

The analytical method for the determination of boscalid and prothioconazole in test biological solutions was successfully validated with a limit of quantification (LOQ) of 12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250) 9.8 mg Prothioconazole/L (diluted by factor 250). No additional confirmatory method or an ILV is required.

Table A 14: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Stock solution	Boscalid	LOQ level (12 g test item/L)	101	1	Range: 100 - 103
		HIGH level (24 g test item/L)	100	3	Range: 95 - 102
		Control	Boscalid not detectable		2 replicates
Stock solution	Prothioconazole	LOQ level (12 g test item/L)	102	1	Range: 01 - 104
		HIGH level (24 g test item/L)	101	3	Range: 96 - 103
		Control	Prothioconazole not detectable		2 replicates

Table A 15: Characteristics for the analytical method used for validation of boscalid and prothioconazole residues in stock solution

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r= 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend
Calibration range	5 to 50 mg Boscalid /L corresponding to 30 % of LOQ to 148 % of higher fortification level	2.5 to 25 mg Prothioconazole /L corresponding to 26 % of LOQ to 128 % of higher fortification level
Assessment of matrix effects is presented	Yes, No significant matrix effect (≥ 20 %) was observed.	Yes, No significant matrix effect (≥ 20 %) was observed.
Limit of quantification	12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250)	12 g test item/L corresponding to 9.8 mg Prothioconazole/L (diluted by factor 250)
Final standard solutions and extracts stability	The stock solutions of the reference items were prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed.	

Conclusion

All validity criteria for the analytical method have been met. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.5.2 Confirmatory method (if required)

No confirmatory method is required.

A 2.1.1.3.6 Analytical method 6

A 2.1.1.3.6.1 Method validation

Comments of zRMS:	The analytical method was validated to verify the concentration of boscalid and prothioconazole in stock solution with LOQ 12 g test item/L corresponding to 16.9 mg boscalid/L and 9.8 mg prothioconazole/L (diluted by factor 250). All validity criteria for
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	the analytical method have been met according to the SANTE/2020/12830, Rev.2 for risk assessment purpose.
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Reference:	KCP 5.1.2/09
Report	Boscalid + Prothioconazole (350 + 200 g/kg - WG): Effects on Terrestrial (Non-Target) Plants: Seedling Emergence and Seedling Growth Test, Stürtz. S., Horstmann, C., 2023b, Study No. 176531086, Sponsor Study No.: UPL/2023/0742.
Guideline(s):	Yes, SANTE/2020/12830, Rev.2 (February 14, 2023)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Duplicate samples of approximately 10 mL from the homogenous stock solution and two samples of the matrix (deionised water) as control solution were taken from the biological study 176531086. The concentrations of the active ingredients Boscalid and Prothioconazole were determined in one sample of each duplicate. The samples were stored deep frozen (≤ -20 °C) until analysis was performed. Analysis was performed by HPLC-UV method.

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. The samples were analysed on the day of processing.

Fortification Procedure:

The test item was dissolved in pure water (sonicated for 5 minutes and stirred for 30 minutes) to get fortified samples of about 12 and 24 g test item /L. Fortified samples were diluted with solvent mixture to match calibration range.

Replicates:

Five independent replicates per fortification level Two independent replicates of matrix blanks.

HPLC-Conditions:

System:	Waters Acquity
Software:	Empower 3
Column:	Kinetex C18 (50 x 2.1 mm; 2.6 μ m)
Mobile Phase:	45 % acetonitrile 55 % HPLC-water + 0.1 % formic acid
Flow Rate:	0.4 mL/min
Temperature:	25°C
Injection Volume:	2 μ L
Detector:	DA- Detector at 191 to 400 nm, monitoring wavelength at 250 nm
Retention Time:	Boscalid: 1.73 minutes Prothioconazole: 2.39 minutes

Results and discussions

The analytical method for the determination of boscalid and prothioconazole in test biological solutions was successfully validated with a limit of quantification (LOQ) of 12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250) 9.8 mg Prothioconazole/L (diluted by factor 250). No additional confirmatory method or an ILV is required.

Table A 16: Recovery results from method validation of boscalid and prothioconazole using the analytical method

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Stock solution	Boscalid	LOQ level (12 g test	101	1	Range: 100 - 103

Matrix	Analyte	Fortification level (g/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
		item/L)			
		HIGH level (24 g test item/L)	100	3	Range: 95 - 102
		Control	Boscalid not detectable		2 replicates
Stock solution	Prothioconazole	LOQ level (12 g test item/L)	102	1	Range: 01 - 104
		HIGH level (24 g test item/L)	101	3	Range: 96 - 103
		Control	Prothioconazole not detectable		2 replicates

Table A 17: Characteristics for the analytical method used for validation of boscalid and prothioconazole residues in stock solution

	Boscalid	Prothioconazole
Specificity	Blank value < 30% LOQ	Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 5 data points r = 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend	Individual calibration data presented Calibration line equation presented 5 data points r= 0.9999 Regression residuals presented in residual plot with random distribution and no visible trend
Calibration range	5 to 50 mg Boscalid /L corresponding to 30 % of LOQ to 148 % of higher fortification level	2.5 to 25 mg Prothioconazole /L corresponding to 26 % of LOQ to 128 % of higher fortification level
Assessment of matrix effects is presented	Yes, No significant matrix effect (≥ 20 %) was observed.	Yes, No significant matrix effect (≥ 20 %) was observed.
Limit of quantification	12 g test item/L corresponding to 16.9 mg Boscalid/L (diluted by factor 250)	12 g test item/L corresponding to 9.8 mg Prothioconazole/L (diluted by factor 250)
Final standard solutions and extracts stability	The stock solutions of the reference items were prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed.	

Conclusion

All validity criteria for the analytical method have been met. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation.

A 2.1.1.3.6.2 Confirmatory method (if required)

No confirmatory method is required.

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

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

~~No new or additional studies have been submitted.~~

A 2.1.2.1.1 Analytical method 1

A 2.1.2.1.1.1 Method validation

Comments of zRMS :	The multi-residue method QuEChERS for determination of residues of boscalid was fully validated on in/on five different matrices of plant origin i.e. wheat grains, wheat straw, lettuce, whole orange and oilseed rape seeds according to SANTE/2020/12830, rev.1: - the determination was performed by LC-MS/MS; - the limit of quantification (LOQ) was 0.01 mg/kg; - recovery/precision: mean recovery at each fortification level was 70-120% with RSD $\leq$ 20%; - linearity: $r^2 \geq 0.99$; the standard calibration range was 0.003 mg/kg to 0.20 mg/kg; - matrix effects was < 20% for wheat grains, lettuce and oilseed rape seeds and thus deemed to be insignificant. Significant matrix effects were observed (>20% suppression) in wheat straw and whole orange. Therefore, matrix-matched standards were used for quantification of Boscalid in all of the investigated matrices. - no interfering peaks observed > 30% of LOQ; It should be noted that ILV is available for this analytical method. According to the SANTE/2020/12830 the method is acceptable for risk assessment and monitoring.
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Reference: KCP 5.2/01

Report Development and Validation of Multi-Residue Method QuEChERS for Determination of Boscalid in Different Matrices of Plant Origin, Rastogi, T., 2022b, Study Number: S22-00980, Sponsor Study Number: UPL/2022/0592

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Summary

An analytical method for the determination of boscalid in different matrices of plant origin was validated with regard to recovery, linearity of detector response, repeatability, specificity, matrix effect, stability of working solutions, limit of quantification and limit of detection. The analytical method fulfils the requirements of guidance document SANTE/2020/12830 Rev. 2 (2023) (risk assessment method). No additional confirmatory method or an ILV is required.

Materials and methods

After preparation of the sample material for analysis (the sample material was thoroughly homogenised in a knife mill using dry ice), lettuce, whole orange and oilseed rape seed matrices were stored at $\leq -18^\circ\text{C}$ (frozen condition) in the dark whereas wheat grain and wheat straw matrices were stored at room temperature in the dark before weighing, fortification and extraction.

In brief, samples of investigated matrices, after addition of water to wheat grains, wheat straw and oilseed rape seeds matrices only, were extracted with acetonitrile. The samples were shaken using a horizontal shaker. A Supel QuECitrate Tube (55227-U) containing mixture of magnesium sulphate, sodium chloride,

sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate was added. The extract was shaken and centrifuged. An aliquot of organic layer was transferred to another tube containing primary secondary amine (PSA). The samples were shaken and centrifuged. The samples were diluted in acetonitrile/water (1/1, v/v) containing 0.1% formic acid prior to the LC-MS/MS analysis for the determination of Boscalid.

Chromatographic and Mass Spectrometric Conditions

HPLC-MS/MS determination, MRM scan, positive ion mode with monitorization of 343→307 ion for quantification and 343→140 ion for confirmation.

HPLC system: 1290 Infinity Binary LC System, Agilent Technologies

Pre-column: Phenomenex C18 4x3mm

Column: Thermo Betasil C18 column: Length: 100 mm, i.d.: 2.1 mm, particle size: 5 µm.

Column oven temperature: 40°C

Injection Volume: 30 µL

Mobile Phase:

Eluent A: Water containing 0.1% (v/v) formic acid

Eluent B: Methanol containing 0.1% (v/v) formic acid

Retention Time: 3.1 minutes (approximately)

Results and discussions

The objective of the study was to develop and validate the multi-residue method QuEChERS for the determination of Boscalid analyte in/on five different matrices of plant origin i.e. wheat grains, wheat straw, lettuce, whole orange and oilseed rape seeds according to the guidance document SANTE/2020/12830, rev. 1 for monitoring with a limit of quantification (LOQ) of 0.01 mg/kg for all matrices.

Table A 3: Recovery results from method validation of boscalid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments
Mass Transition: 343 m/z → 307 m/z (Quantification)							
Wheat grains	Boscalid	0.01	98.0	2.6	94.9	5.1	Range: 93.8 - 101
		0.1	91.7	5.0			Range: 86.2 - 96.0
Wheat straw	Boscalid	0.01	90.3	7.5	87.7	7.0	Range: 79.2 - 93.9
		0.1	85.0	5.4			Range: 78.1 - 89.5
Lettuce	Boscalid	0.01	99.5	2.4	96.2	4.1	Range: 95.8 - 102
		0.1	92.9	1.4			Range: 91.4 - 94.8
Whole orange	Boscalid	0.01	93.8	2.9	94.3	3.8	Range: 90.2 - 97.0
		0.1	94.9	4.9			Range: 90.2 - 102
Oilseed rape seed	Boscalid	0.01	80.2	3.8	80.2	3.2	Range: 76.8 - 83.4
		0.1	80.3	3.0			Range: 77.8 - 82.8
Mass Transition: 343 m/z → 140 m/z (Confirmation)							
Wheat grains	Boscalid	0.01	95.3	3.1	93.8	4.4	Range: 92.4 - 100
		0.1	92.3	5.3			Range: 86.2 - 96.8
Wheat straw	Boscalid	0.01	90.3	6.2	87.4	6.4	Range: 81.2 - 95.6
		0.1	84.5	84.5			Range: 77.8- 87.8
Lettuce	Boscalid	0.01	98.7	2.6	96.0	3.5	Range: 96.0 - 102

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments
		0.1	93.4	1.5			Range: 91.4 - 94.9
Whole orange	Boscalid	0.01	97.4	1.8	96.9	3.3	Range: 95.4 - 100
		0.1	96.5	4.6			Range: 93.4 - 104
Oilseed rape seed	Boscalid	0.01	82.0	3.0	80.2	4.3	Range: 79.0 - 84.0
		0.1	78.3	4.4			Range: 74.2 - 82.2

No residue above 30% of the LOQ level was detected in any control sample extract

Recoveries are without any blank correction

Analyte: Boscalid; Final determination as: Boscalid; Residues calculated as: Boscalid

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 analyzed against freshly prepared calibration standards. One mass transition (343 m/z → 307 m/z) 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 13 days in the dark.

Table A 4: Stability of Analytes in Sample Extracts

Matrix	Fortification level (mg/kg)	Days of storage *	Mean Recovery after storage, n = 5 (%)	Rel. Std. Dev., n = 5 (%)
Wheat Grains	0.10	13	92.1	4.9
Wheat Straw	0.10	13	74.7	2.6
Lettuce	0.10	15	104	1.6
Whole Orange	0.10	15	106	3.8
Oilseed Rape Seeds	0.10	15	82.3	4.7

* from the day of completion of sample work-up to the day of LC-MS/MS analysis for 2nd measurement

Table A 5: Characteristics for the analytical method used for validation of boscalid residues in plants

	MRM 1 (343 m/z → 307 m/z)	MRM 2 (343 m/z → 140 m/z)
Specificity	Mass spectrum is provided Blank value < 30% LOQ	Mass spectrum is provided Blank value < 30% LOQ
Calibration (type, number of data points)	Single determination Calibration line equation presented Linear, 1/x - weighting r ² ≥ 0.99 6 data points Matrix-matched	Single determination Calibration line equation presented Linear, 1/x - weighting r ² ≥ 0.99 6 data points Matrix-matched
Calibration range	Calibration range 0.15 – 10.00 ng/mL, six concentration levels Equivalent to 0.003 to 0.20 mg/kg in injected samples with < 30% of LOQ and ≥ + 20% of the highest analyte concentration	Calibration range 0.15 – 10.00 ng/mL, six concentration levels Equivalent to 0.003 to 0.20 mg/kg in injected samples with < 30% of LOQ and ≥ + 20% of the highest analyte concentration
Assessment of matrix effects is presented	Yes Insignificant for wheat grain, lettuce and oilseed rape seeds Significant for wheat straw and whole orange	Yes Insignificant for wheat grain, lettuce and oilseed rape seeds Significant for wheat straw and whole orange

	MRM 1 (343 m/z → 307 m/z)			MRM 2 (343 m/z → 140 m/z)		
	Wheat Grain	0.50 (LOQ)	(-) 2.9	Wheat Grain	0.50 (LOQ)	(+) 2.9
		5.0 (10 X LOQ)	(+) 1.6		5.0 (10 X LOQ)	(+) 0.71
	Wheat Straw	0.50 (LOQ)	(-) 41	Wheat Straw	0.50 (LOQ)	(-) 37
		5.0 (10 X LOQ)	(-) 34		5.0 (10 X LOQ)	(-) 35
	Lettuce	0.50 (LOQ)	(-) 34	Lettuce	0.50 (LOQ)	(-) 1.1
		5.0 (10 X LOQ)	(-) 0.96		5.0 (10 X LOQ)	(-) 1.4
	Whole Orange	0.50 (LOQ)	(-) 21	Whole Orange	0.50 (LOQ)	(-) 26
		5.0 (10 X LOQ)	(-) 25		5.0 (10 X LOQ)	(-) 24
	Oilseed rape seeds	0.50 (LOQ)	(-) 14	Oilseed rape seeds	0.50 (LOQ)	(-) 16
		5.0 (10 X LOQ)	(-) 16		5.0 (10 X LOQ)	(-) 14
Limit of determination/quantification	LOQ = 0.01 mg/kg LOD = 0.003 mg/kg			LOQ = 0.01 mg/kg LOD = 0.003 mg/kg		
Stability of Working Solution	Boscalid was found to be stable for 16 days when prepared in methanol and stored at typically 1°C to 10°C in the dark.					
Stability of Final Extracts	Boscalid was found to be stable in final extracts of all matrices for at least 13 days when stored at typically 1°C to 10°C in the dark.					

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring for the determination of Boscalid in five different matrices of plant origin i.e. wheat grains, wheat straw, lettuce, whole orange and oilseed rape seeds with the tested LOQ of 0.01 mg/kg and meets the requirements of SANTE/2020/12830, rev. 2.

A 2.1.2.1.1.2 Independent laboratory validation

Comments of zRMS :	The analytical method of Rastogi, T. (2022b, Study Number: S22-00980) was independently validated with the LOQ of 0.01 mg/kg in wheat (grain and straw), lettuce, whole orange and oilseed rape seed according to the guidance document SANTE/2020/12830, rev. 1.
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Reference: KCP 5.2/02

Report Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Boscalid in Different Matrices of Plant Origin, Gustloff, C., 2022, Study Number: S22-05632, Sponsor Study Number: UPL/2022/0875

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Summary

To independently validate (ILV) the multi-residue method QuEChERS for the determination of Boscalid in wheat (grain and straw), lettuce, whole orange and oilseed rape seed in accordance to guidance document SANTE/2020/12830, rev.1 for risk assessment and/or monitoring.

Materials and methods

Samples of investigated matrices, after addition of water to wheat grains, wheat straw and oilseed rape

seeds matrices only, were extracted with acetonitrile. The samples were shaken using horizontal shaker. QuEChERS salt mixture containing magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate was added. The extract was shaken and centrifuged. An aliquot of organic layer was transferred to another tube containing primary secondary amine (PSA) and magnesium sulphate (MgSO₄). The samples were shaken and centrifuged. The samples were diluted in acetonitrile/water (1/1, v/v) containing 0.1% formic acid prior to the LC-MS/MS analysis for the determination of Boscalid.

Chromatographic conditions

HPLC system	1260 Infinity Binary LC System, Agilent Technologies (HPLC, ≤ 600 bar)
Pre-column	SecurityGuard™ ULTRA Cartridge(C18) (2 mm x 2.1 mm, - µm, Phenomenex, Art. No. AJ0-8782)
Column	Betasil C18 (100 mm x 2.1 mm, 5 µm, Thermo Scientific, Art. No. 70105-102130)
Column oven temperature	40 °C
Injection volume	30 µL
Mobile phases	Eluent A: Methanol containing 0.1 % formic acid (v/v) Eluent B: Water containing 0.1 % formic acid (v/v)

Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	30	70	600
	1.0	30	70	600
	1.5	70	30	600
	3.0	70	30	600
	3.5	90	10	600
	5.0	90	10	600
	5.5	30	70	600
	7.0	30	70	600

Vivert valve to mass spectrometer from 2.5 min to 4.5 min

Retention time Boscalid: approx. 3.1 min

Mass spectrometric conditions

MS system	TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
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 25 (arbitrary units)	Gas flow 1 (GS1)		Zero-grade air set at 40 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 7 (arbitrary units)		Gas flow 2 (GS2)		Zero-grade air set at 60 (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]
Boscalid	343 → 307# (pos)	120	10	43	36	100
	343 → 140 (pos)	46	10	25	12	100

Results and discussions

Test method for the determination of boscalid and its metabolites in plant matrices was independently validated according to the guidance document SANTE/2020/12830, rev. 1.

Table A 6: Recovery results from independent laboratory validation of Test method for boscalid residues in plant matrices

Boscalid							
Mass Transition	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Lettuce							
m/z 343→307*	0.01	96, 95, 89, 103, 97	96	5.4	5	98	4.3
	0.1	100, 103, 97, 96, 99	99	2.7	5		
m/z 343→140	0.01	99, 97, 91, 109, 101	99	6.8	5	100	4.9
	0.1	101, 105, 99, 98, 101	101	2.9	5		
Wheat (grain)							
m/z 343→307*	0.01	89, 80, 90, 84, 88	86	5.0	5	85	7.9
	0.1	84, 86, 77, 74, 97	84	11	5		
m/z 343→140	0.01	89, 83, 92, 86, 94	89	5.2	5	87	8.7
	0.1	86, 85, 76, 77, 101	85	12	5		
Orange (whole)							
m/z 343→307*	0.01	112, 101, 98, 98, 103	102	5.5	5	101	4.0
	0.1	97, 102, 101, 100, 100	100	1.9	5		
m/z 343→140	0.01	106, 104, 104, 105, 105	105	1.0	5	102	2.6
	0.1	99, 100, 101, 100, 100	100	1.0	5		
Wheat (straw)							
m/z 343→307*	0.01	83, 81, 70, 74, 72	76	7.5	5	76	7.6
	0.1	66, 77, 82, 74, 82	76	8.6	5		
m/z 343→140	0.01	75, 99, 86, 67, 65	79	18	5	77	13
	0.1	68, 80, 77, 72, 82	76	7.5	5		
Oilseed rape (seeds)							
m/z 343→307*	0.01	79, 79, 74, 74, 74	76	3.8	5	78	4.3
	0.1	82, 79, 81, 82, 75	80	3.5	5		
m/z 343→140	0.01	80, 84, 84, 65, 76	78	10	5	79	7.3
	0.1	81, 79, 80, 84, 75	80	3.9	5		

* Proposed to be used for quantification

Table A 7: Characteristics for the analytical method used for independent laboratory validation of test method 1 for boscalid residues in animal matrices

Selectivity and Specificity	Demonstrated by validation of two mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.
Calibration	Matrix-matched calibration standards A minimum of five concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.15 ng/mL to 10 ng/mL Corresponding mass fraction range: 0.003 mg/kg to 0.20 mg/kg Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract The validated range does not exceed two orders of magnitude
Quantification	Linear regression with 1/x weighting. Regression residuals randomly distributed

	Coefficients of determination (R ²) ≥ 0.99									
Limit of determination/quantification	LOQ = 0.01 mg/kg for boscalid in wheat (grain and straw), lettuce, whole orange and oilseed rape seed LOD = 0.003 mg/kg for all matrices (30 % of the LOQ)									
Accuracy and Precision	Five fortifications at 0.01 mg/kg (LOQ) Five fortifications at 0.1 mg/kg (10x LOQ) Mean recoveries for both evaluated mass comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1: <table><tr><td>Concentration level (mg/kg)</td><td>Range of mean Recoveries (%)</td><td>Precision, RSD (%)</td></tr><tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr><tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr></table>	Concentration level (mg/kg)	Range of mean Recoveries (%)	Precision, RSD (%)	≤ 0.01	60 - 120	≤ 30	> 0.01 - ≤ 0.1	70 - 120	≤ 20
Concentration level (mg/kg)	Range of mean Recoveries (%)	Precision, RSD (%)								
≤ 0.01	60 - 120	≤ 30								
> 0.01 - ≤ 0.1	70 - 120	≤ 20								
Stability of Analyte in Standard Solutions	Within ± 10 % for 229 days when prepared in methanol and stored at typically 1 °C to 10 °C in the dark.									
Stability of Analyte in Sample Extracts	Recoveries within 70% - 120% in all matrix extracts for at least 13 days for wheat (grain and straw) and oilseed rape (seeds) and for at least 7 days for whole orange and lettuce when stored at typically 1 °C to 10 °C in the dark.									

Conclusion

Primary validation and independent laboratory validation were carried out at different locations and by different study personnel. No addition or modification to the original method other than optimization of instrumental parameters was done.

No communication with the method developers or others familiar with the method was necessary to carry out the analysis.

The method was found to be valid according to the guidance documents SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring

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

A 2.1.2.2.1 Analytical method 1

A 2.1.2.2.1.1 Method validation

Comments of zRMS:	Three analytical methods for determination of residues of boscalid, M510F01, M510F52 and M510F53 were fully validated on in/on matrices of animal origin such as bovine meat (as muscle), bovine fat, milk, poultry's eggs, bovine liver and bovine kidney according to SANTE/2020/12830, rev.1 with LOQ of 0.01 mg/kg in each matrices. The definition of residue of boscalid for monitoring method and risk assessment method in the food/feed of animal origin matrices are as follows: a. Definition of residue for monitoring: Food/Feed of animal origin: muscle, fat, milk and eggs: parent boscalid only (<i>Test Method 1</i>). liver and kidney: Sum of boscalid and its hydroxyl metabolite M510F01 (free and conjugated), expressed as boscalid (<i>Test Method 2</i>). b. Definition of residue for exposure/risk assessment: Food/Feed of animal origin: muscle, fat, milk and eggs: parent boscalid only (<i>Test Method 1</i>). liver and kidney: Sum of boscalid and its hydroxyl metabolite M510F01 (free and conjugated), expressed as boscalid (<i>Test Method 2</i>). liver: Sum of boscalid and its hydroxyl metabolite M510F01 (free and conjugated) and its
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	bound residues (measured as M510F53 and M510F52), expressed as boscalid (<i>Test Method 3</i>). It should be noted that ILV is available for this analytical method. According to the SANTE/2020/12830 the method is acceptable for risk assessment and monitoring.
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Reference: KCP 5.2/03

Report Development and Validation of Analytical Methods for Determination of Boscalid and its Metabolite M510F01 and Bound Residues in Different Matrices of Animal Origin, Rastogi, T. 2023, Study No. S22-00981, Sponsor study No.UPL/2022/2043

Guideline(s): SANTE/2020/12830 Rev.2 Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post -approval Control and Monitoring Purposes, February 14, 2023

Deviations: No

GLP: Yes

Acceptability: Yes

Summary

An analytical method for the determination of boscalid and its Metabolite M510F01 and bound residues in different matrices of animal origin such as bovine meat (as muscle), bovine fat, milk, poultry's eggs, bovine liver and bovine kidney was validated with regard to recovery, linearity of detector response, repeatability, specificity, matrix effect, stability of working solutions, limit of quantification and limit of detection. The analytical method fulfils the requirements of guidance document SANTE/2020/12830, rev.1 (risk assessment method). No additional confirmatory method or an ILV is required.

Materials and methods

Material: Reference item:

- **Boscalid**; CAS No.: 188425-85-6, Batch/Lot No.: G1040010, Purity: 99.02 % (g/g), Expiry Date: 25 Oct 2023
- **Metabolite M510F01**; CAS No.: 661463-87-2 ,Batch/Lot No.: EPP/RH 2610. Purity: 91.05 % (g/g), Expiry Date: 18 July 2023
- **Metabolite M510F52**: CAS No.: na, Batch/Lot No.: EPP/AZ 119, Purity: 99.16 % (g/g), Expiry Date: 18 July 2023
- **Metabolite M510F53**: CAS No.: na, Batch/Lot No.: EPP/AZ 118, Purity: 99.61 % (g/g), Expiry Date: 18 July 2023

Test Methods

Method 1: Determination of Boscalid only in muscle (bovine meat), bovine whole milk, bovine fat and poultry's eggs.

Method 2: Determination of Boscalid and its hydroxyl metabolite M510F01 and potential conjugates in bovine liver and bovine kidney.

Method 3: Determination of bound residues of M510F53 and M510F52 in bovine liver.

Preparation of Standard Solutions

Stock solutions of the analytes were prepared by dissolving a weighted amount of the test / reference items into HPLC grade methanol with the aid of an ultrasonic bath.

The stock solutions of all analytes were further diluted to tune solutions, fortification solutions and to intermediate standard solutions. The tune solutions of M510F01 were used for optimizing the LC-MS/MS instrument parameters. The fortification solutions were used to fortify the investigated matrix specimens, and the intermediate standard solutions were further used for preparation of calibration standards in solvent and matrix-matched calibration standards. Matrix-matched calibration solutions were prepared by diluting the intermediate standard solutions into the final extracts of control (untreated) samples of all

investigated matrices (at least 90 % matrix amount).

All solutions except stock solutions were stored refrigerated (typically 1 °C to 10 °C) in brown glass vials in the dark. The stock solution was stored under frozen condition (typically ≤ -18 °C) in brown glass vials

Chromatographic and Mass Spectrometric Conditions

Chromatographic conditions for Boscalid, M510F01, M510F52 and M510F53						
HPLC system	1290 Infinity Binary LC System, Agilent Technologies					
Pre-column	Phenomenex C ₁₈ 4x3 mm					
Column	Thermo Betasil C18 column: Length: 100 mm, i.d.: 2.1 mm, particle size: 5.0 µm.					
Column oven temperature	40 °C					
Injection volume	30 µL					
Mobile phases	Eluent A: Water containing 0.1 % (v/v) formic acid Eluent B: Methanol containing 0.1 % (v/v) formic acid					
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]		
	0.00	70	30	600		
	1.00	70	30	600		
	1.50	30	70	600		
	3.00	30	70	600		
	3.50	10	90	600		
	5.00	10	90	600		
	5.50	70	30	600		
	7.00	70	30	600		
Divert valve	0.0 min to 7.0 min to MS					
Retention time(s)	Boscalid: approx. 3.2 min M510F01: approx. 2.4 min M510F52: approx. 3.0 min M510F53: approx. 2.8 min					
Mass spectrometric conditions for Boscalid ,M510F01, M510F52 and M510F53						
MS system	Applied Biosystems	API 5500 Q-Trap LC/MS System, SCIEX				
Ionisation type	Electrospray ionisation	(ESI, Turbo Spray)				
Scan type	MS/MS, Multiple R	action Monitoring (MRM)				
Polarity	Positive ion mode					
Capillary voltage (IS)	4500 V	Ion spray turbo heater (TEM)		550 °C		
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)	Gas flow 1 (GS1)		Nitrogen set at 40 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at Medium (arbitrary units)	Gas flow 2 (GS2)		Nitrogen set at 60 (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]
Boscalid	343 → 307 [#]	91	10	27	26	150
	343 → 271	91	10	43	22	150
M510F01	359 → 323 [#]	106	10	25	24	150

	359 → 140	106	10	25	12	150
M510F52	232 → 169 [#]	76	10	29	14	150
	232 → 204	76	10	15	16	150
M510F53	246 → 169 [#]	86	10	35	14	150
	246 → 204	86	10	19	18	150
Chromatographic conditions for M510F52						
HPLC system	Agilent Infinity 1290 Series HPLC system (vacuum solvent degasser, binary HPLC pump, multisampler, multicolumn thermostat)					
Pre-column	Phenomenex C ₁₈ 4x3 mm					
Column	Thermo Betasil C18 column: Length: 100 mm, i.d.: 2.1 mm, particle size: 5.0 μm.					
Column oven temperature	40 °C					
Injection volume	30 μL					
Mobile phases	Eluent A: Water containing 0.1 % (v/v) formic acid; Eluent B: Methanol containing 0.1 % (v/v) formic acid					
Gradient	Time [min]	% Eluent A	% Eluent B		Flow [μL/min]	
	0.00	70	30		600	
	1.00	70	30		600	
	1.50	30	70		600	
	3.00	30	70		600	
	3.50	10	90		600	
	5.00	10	90		600	
	5.50	70	30		600	
	7.00	70	30		600	
Retention time(s)	M510F52: approx. 2.9 min					
Mass spectrometric conditions for M510F52						
MS system	Sciex 4000 Triple Quadrupole MS/MS system					
Ionisation type	Electrospray ionisation (ESI, Turbo Spray)					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Polarity	Positive ion mode					
Capillary voltage (IS)	4500 V		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]
M510F52	232 → 169 [#]	30	10	29	14	150
	232 → 204	30	10	15	16	150

[#] proposed (and used) for quantification but both of the mass transitions listed can be used for quantification

Results and discussions

Table 20: Recovery results from method validation of Boscalid using the analytical method for test method 1

Test Method 1							
Boscalid							
Matrix	Fortification level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev (%)
Mass Transition 343 m/z→ 307 m/z(Proposed for Quantification)							
Bovine Milk	0.010	102,97.2,93.8,98.4,92.0	96.7	4.1	5	99.7	5.2
	0.1	109,105,103,98.2,98.6	103	4.5	5		
Poultry eggs	0.010	109,110,108,101,93.8	104	6.5	5	107	5.6
	0.1	116,111,107,109,110	111	2.9	5		
Bovine Meat	0.010	95.4,99.6,100,96.2,102	98.6	2.7	5	98.5	2.8
	0.1	104,96.2,96.8,98.0,96.8	98.3	3.2	5		
Bovine Fat	0.010	82.0,81.2,88.8,81.0,86.0	93.8	4.1	5	88.9	7.9
	0.1	95.4,87.6,101,98.0,88.4	94.0	6.2	5		
Mass Transition 343 m/z→ 271 m/z(Proposed for Confirmation)							
Bovine Milk	0.010	101,97.0,92.2,96.2,92.2	95.6	3.7	5	97.4	3.9
	0.1	105,100,98.2,96.8,95.4	99.1	3.6	5		
Poultry eggs	0.010	109,104,107,102,89.6	102	7.4	5	107	6.9
	0.1	116,112,106,112,111	111	3.3	5		
Bovine Meat	0.010	103,101,98.4,105,106	103	3.1	5	101	3.9
	0.1	108,96.6,98.0,99.6,97.8	99.9	4.4	5		
Bovine Fat	0.010	73.3,78.0,76.2,79.2,84.8	78.3	5.4	5	86.6	12
	0.1	97.9,89.8,100,99.6,87.2	95.0	6.4	5		

Table 21: Recovery results from method validation of Boscalid using the analytical method for test method 2

Table 21. Recovery Results from method validation of Boscalid using the analytical method for test method 2							
	Test Method 2						
	Boscalid						
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
	Mass Transition 343 m/z → 307 m/z (Proposed for Quantification)						
Bovine Liver	0.010	105, 91.8, 108, 102, 106	103	6.2	5	99.6	5.5
	0.10	100, 95.4, 96.8, 96.4, 94.6	96.7	2.2	5		
Bovine Kidney	0.010	104, 103, 84.4, 95.6, 96.8	96.8	8.2	5	93.6	7.4
	0.10	96.7, 89.0, 92.6, 88.6, 85.0	90.4	4.9	5		
	Mass Transition 343 m/z → 271 m/z (Proposed for Confirmation)						
Bovine Liver	0.010	106, 96.8, 106, 103, 106	104	3.8	5	99.7	5.2
	0.10	100, 93.4, 96.8, 95.4, 93.4	95.9	3.0	5		
Bovine Kidney	0.010	105, 102, 85.4, 93.0, 96.6	96.4	8.1	5	93.2	7.4
	0.10	96.5, 89.4, 91.6, 87.2, 84.8	89.9	4.9	5		

M510F01							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition 359 m/z → 323 m/z (Proposed for Quantification)							
Bovine Liver	0.010	104, 87.0, 104, 102, 105	100	7.5	5	93.6	9.4
	0.10	89.6, 87.6, 87.6, 83.4, 86.4	86.9	2.6	5		
Bovine Kidney	0.010	95.5, 104, 95.0, 87.2, 93.4	95.1	6.4	5	92.2	6.2
	0.10	94.1, 87.2, 93.4, 85.2, 86.8	89.3	4.6	5		
Mass Transition 359 m/z → 140 m/z (Proposed for Confirmation)							
Bovine Liver	0.010	93.3, 81.8, 94.4, 90.0, 93.8	90.7	5.8	5	88.0	5.3
	0.10	87.6, 83.0, 87.0, 83.2, 85.8	85.3	2.5	5		
Bovine Kidney	0.010	98.1, 105, 84.4, 81.0, 87.6	91.2	11	5	88.7	8.7
	0.10	91.0, 82.8, 89.4, 81.6, 85.8	86.1	4.7	5		

Table 22: Recovery results from method validation of M510F52 using the analytical method for test method 3

Test Method 3							
M510F52							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition 232 m/z → 169 m/z (Proposed for Quantification)							
Bovine Liver	0.010	76.6, 72.0, 73.0, 78.0, 74.6	74.8	3.3	5	80.1	15
	0.10	87.9, 111, 70.8, 79.6, 77.2	85.3	18	5		
Mass Transition 232 m/z → 204 m/z (Proposed for Confirmation)							
Bovine Liver	0.010	76.2, 75.8, 70.8, 76.4, 76.2	75.1	3.2	5	80.9	14
	0.10	87.4, 110, 73.2, 81.6, 81.2	86.6	16	5		
M510F53							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition 246 m/z → 169 m/z (Proposed for Quantification)							
Bovine Liver	0.010	90.4, 89.4, 75.2, 97.8, 77.8	86.1	11	5	96.6	14
	0.10	95.3, 111, 111, 101, 117	107	8.3	5		
Mass Transition 246 m/z → 204 m/z (Proposed for Confirmation)							
Bovine Liver	0.010	94.9, 89.4, 78.2, 101, 75.2	87.7	12	5	97.3	15
	0.10	90.7, 115, 111, 100, 118	107	11	5		

Table 23: Characteristics for the analytical method used for validation of Boscalid & metabolites in water animal matrices

Principle of the Analytical Procedure	Test Method 1 - Extraction: Addition of water (if necessary) and extraction with acetonitrile - Liquid/Liquid Partition: Addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation - Clean-up: Dispersive SPE with primary/secondary amine (PSA) - Dilution: in acetonitrile/water (1/1, v/v) containing 0.1% formic acid prior to the LC-MS/MS analysis - Quantification: LC-MS/MS Test method 2 - Extraction: Addition of water and extraction with acetonitrile - Liquid/Liquid Partition: Addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation - Hydrolysis: 0.1 M hydrochloric acid, heating under reflux for 2 hours at 140°C - Neutralization: 5 M sodium hydroxide - Reconstitution: Acetonitrile - Liquid/Liquid Partition: Addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation - Clean-up: Dispersive SPE with primary/secondary amine (PSA) - Dilution: in acetonitrile/water (1/1, v/v) containing 0.1% formic acid prior to the LC-MS/MS analysis - Quantification: LC-MS/MS Test Method 3 - Extraction: With Acetonitrile - Microwave: 30 minutes at 170 °C with 0.1 % formic acid (for M510F52) or acetic acid (for M510F53) - Dilution: in water prior to the LC-MS/MS analysis - Quantification: LC-MS/MS
Selectivity and Specificity:	Demonstrated by validation of two mass transitions for each analyte Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were equal or below of what would correspond to an analyte level of 30 % of the LOQ.
Matrix Effects	Mean Matrix effects did not exceed ± 20 % (for all matrix : in milk, eggs, meat and fat, liver and kidney, and liver) and were deemed to be insignificant for boscalid and its metabolites. Nevertheless, matrix-matched standards were used for quantification throughout the study. The effect of matrix on the LC-MS/MS response was assessed by comparing peak area of matrix-matched standards of at least 95 % matrix amount with solvent standards within the same calibration range.
Stability	Stability of Analyte(s) in Final Extracts: The interval from preparation of the final extracts to injection did not exceed 7 days. Stability of Analyte(s) in Working Solutions Stock solutions prepared in methanol were stored at typically frozen conditions ($\leq -18^{\circ}\text{C}$) in the dark (45 days for boscalid and M510F01 and 51 days for M510F52 and M510F53). This was sufficient to cover the length of time working solutions in methanol were used in this study. After this time freshly prepared dilutions of the stored stock solutions were compared to freshly prepared solutions by at least fivefold injection. One mass transition per analyte was evaluated. The mean peak areas of the stored working solutions were within ± 10 % of the mean peak areas of the freshly prepared solutions indicating that all working solutions are stable when stored at frozen conditions ($\leq -18^{\circ}\text{C}$) in the dark for the respective time interval.

Linearity	Matrix-matched calibration standards A minimum of six (6) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.150 ng/mL to 10.0 ng/mL Corresponding mass fraction range: 0.003 mg/kg to 0.20 mg/kg Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract The validated range does not exceed two (2) orders of magnitude The linearity of the three methods was determined by measuring the detector response (peak area) versus the concentration of a series of at least 6 calibration standard solutions and covered a maximum of two orders of magnitude. The analytical calibration covered the range from 0.150 ng/L to 10.0 ng/L (30% of the LOQ to 20% above the highest level). Range: 0.150 to 10.0 ng/L. $r>0.99$									
Limit of determination/quantification	The LOQ of the method is defined as the lowest analyte concentration at which the methodology has been successfully validated. The LOD was defined as the lowest calibration standard (≤ 30 % of the LOQ). LOQ = 0.01 mg/kg for boscalid (bovine milk, poultry eggs, bovine meat and bovine fat) and for boscalid equivalent (for bovine liver, kidney and liver) LOD = 0.003 mg/kg for boscalid (bovine milk, poultry eggs, bovine meat and bovine fat) and for boscalid equivalent (for bovine liver, kidney and liver)									
Accuracy and Precision	The accuracy and precision of the methods during sample analysis were considered to be acceptable the mean recoveries at each fortification level were in the range of 60 % - 120 % with the RSD below 30 % at the LOQ and in the range of 70 % - 120 % with the RSD below 20 % at high dose level in each matrices. <table><tr><th>Concentration level (mg/kg)</th><th>Range of mean Recoveries (%)</th><th>Precision, RSD (%)</th></tr><tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr><tr><td>$> 0.01 - \leq 0.1$</td><td>70 - 120</td><td>≤ 20</td></tr></table>	Concentration level (mg/kg)	Range of mean Recoveries (%)	Precision, RSD (%)	≤ 0.01	60 - 120	≤ 30	$> 0.01 - \leq 0.1$	70 - 120	≤ 20
Concentration level (mg/kg)	Range of mean Recoveries (%)	Precision, RSD (%)								
≤ 0.01	60 - 120	≤ 30								
$> 0.01 - \leq 0.1$	70 - 120	≤ 20								

Conclusion

The three methods were successfully validated for the determination of Boscalid and related metabolites in/on different matrices of animal origin such as bovine meat (as muscle), bovine fat, milk, poultry's eggs, bovine liver and bovine kidney from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg (expressed as Boscalid equivalent) according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.

A 2.1.2.2.1.2 Independent laboratory validation

Comments of zRMS:	The analytical methods (Test methods 1, 2 and 3) of Rastogi, T. (2022, Study No. S22-00981) for the determination of boscalid and its metabolites in animal matrices were independently validated with the LOQ of 0.01 mg/kg in each analytes according to the guidance document SANTE/2020/12830, rev. 1.
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Reference:

KCP 5.2/04

Report

Independent Laboratory Validation of Analytical Methods for Determination of Boscalid and its Metabolite M510F01 and Bound Residues of the Metabolites M510F52 and M510F53 in Different Matrices of Animal Origin, Spielvogel Ch., Timmann, A., 2023, Eurofins Agrosience Services EcoChem GmbH Eutinger Str. 24 75223 Niefern-Öschelbronn Germany, Study No. S22-05633 (EAG-2202V), Sponsor Study No. UPL/2023/0500.

Guideline(s):	Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The principle of the analytical procedure is in line with the primary method:

Test method 1

QuEChERS method: Boscalid residues were extracted with acetonitrile after addition of water if needed. After addition of magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate, the samples have been centrifuged.

Clean up step only for bovine fat matrix was carried out as follows: the upper organic layer was transferred into centrifuge tube and frozen out for 2 hours followed by centrifugation.

Purification of an aliquot of the acetonitrile extract by dispersive SPE with primary/secondary amine (PSA)

The samples were then analysed with HPLC-MS/MS.

Test method 2

Boscalid and M510F01 residues were extracted with acetonitrile after addition of water if needed.

After addition of magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate, the samples have been centrifuged.

A step with Hydrolysis by reflux with hydrochloric acid was then followed by neutralization.

Another Liquid/Liquid partition identical to the first one followed.

The samples were then analysed with HPLC-MS/MS.

Test method 3

M510F52 and M510F53 residues were extracted from liver with acetonitrile

Microwave treatment was carried out with formic acid for M510F52 and with acetic acid for M510F53.

The samples were then analysed with HPLC-MS/MS.

Chromatographic conditions for determination of Boscalid, M510F01:

HPLC system: 1260 Infinity Binary LC System, Agilent Technologie

Column: Betasil C18(100 mm x 2.1 mm, 5 µm).

Column oven temperature: 40 °C

Injection volume: 30 µL

Mobile phases:

Eluent B: Water+ 0.1 % v/v formic acid

Eluent A: Methanol+ 0.1 % v/v formic acid

Retention time: Boscalid: approx. 3.62 min

M510F01 approx. 2.93 min

MS system: SCIEX QTRAP API 5500 System, SCIEX (Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer). Electrospray ionisation (ESI, TurboIonSpray). Positive ion mode. MS/MS, Multiple Reaction Monitoring (MRM). Mass transition monitored:

Boscalid: 343→307 (quantitation) and 343→271 (confirmation)

M510F01: 359→323 (quantitation) and 359→140 (confirmation)

Chromatographic conditions for determination of M510F52 and M510F53:

HPLC system: 1260 Infinity Binary LC System, Agilent Technologie

Column: Thermo Betasil C18(100 mm x 2.1 mm, 5 µm).

Column oven temperature: 40 °C

Injection volume: 10 µL

Mobile phases:

Eluent A: Methanol+ 0.1 % v/v formic acid

Eluent B: Water+ 0.1 % v/v formic acid
Retention time: M510F52 approx. 3.52 min
M510F53 approx. 3.34 min

MS system: SCIEX Triple Quadrupole MS/MS system55000, SCIEX (Triple Quadrupole LC/MS/MS Mass Spectrometer). Electrospray ionisation (ESI, TurboIonSpray). Positive ion mode. MS/MS, Multiple Reaction Monitoring (MRM). Mass transition monitored:
M510F52: 232→169 (quantitation) and 232→204 (confirmation)
M510F53: 246→169 (quantitation) and 246→204 (confirmation)

Results and discussions

Test methods 1, 2 and 3 for the determination of boscalid and its metabolites in animal matrices were independently validated according to the guidance document SANTE/2020/12830, rev. 1.

Table A 23: Recovery results from independent laboratory validation of Test method 1 for boscalid

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Milk	Boscalid	0.01	95	4	Range: 91-100
		0.1	92	3	Range: 89-96
Overall			93	4	n=10
Eggs	Boscalid	0.01	104	3	Range: 100-110
		0.1	100	2	Range: 97-102
Overall			102	4	n=10
Meat	Boscalid	0.01	105	3	Range: 101-108
		0.1	99	1	Range: 98-101
Overall			102	4	n=10
Fat	Boscalid	0.01	93	2	Range: 90-96
		0.1	92	3	Range: 89-95
Overall			92	2	n=10
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Milk	Boscalid	0.01	97	4	Range: 93-100
		0.1	93	3	Range: 90-97
Overall			95	3.4	n=10
Eggs	Boscalid	0.01	105	3	Range: 102-109
		0.1	102	2	Range: 100-105
Overall			104	3	n=10
Meat	Boscalid	0.01	106	4	Range: 98-109
		0.1	103	4	Range: 101-104
Overall			104	3	n=10
Fat	Boscalid	0.01	95	4	Range: 90-102
		0.1	94	2	Range: 91-97
Overall			95	3	n=10

Table A 24: Recovery results from independent laboratory validation of Test method 2 for boscalid and M510F01

Boscalid and M510F01					
Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Liver	Boscalid	0.01	106	3	Range: 101-108
		0.1	108	1	Range: 107-109
Overall			107	2	n=10
Kidney	Boscalid	0.01	107	3	Range: 104-111
		0.1	101	3	Range: 99-105
Overall			104	4	n=10
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Liver	Boscalid	0.01	108	3	Range: 105-112
		0.1	109	2	Range: 107-112
Overall			108	2.1	n=10
Kidney	Boscalid	0.01	105	4	Range: 98-110
		0.1	104	2	Range: 102-108
Overall			105	3	n=10
Mass Transition m/z 359 to 323 (Proposed for Quantification)					
Liver	M510F01	0.01	105	10	Range: 90-113
		0.1	102	2	Range: 99-104
Overall			103	7	n=10
Kidney	M510F01	0.01	106	3	Range: 104-111
		0.1	102	1	Range: 100-103
Overall			104	3.1	n=10
Mass Transition m/z 359 to 140 (Proposed for Confirmation)					
Liver	M510F01	0.01	96	3	Range: 94-100
		0.1	102	2	Range: 99-105
Overall			99	4	n=10
Kidney	M510F01	0.01	102	5	Range: 94-105
		0.1	100	2	Range: 99-103
Overall			101	3	n=10

Table A 25: Recovery results from independent laboratory validation of Test method 3 for M510F52 and M510F53

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 232 to 169 (Proposed for Quantification)					
Liver	M510F52	0.01	88	7	Range: 85-94
		0.1	94	9	Range: 83-103
Overall			91	8	n=10
Mass Transition m/z 232 to 204 (Proposed for Confirmation)					
Liver	M510F52	0.01	82	5	Range: 76-87

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
		0.1	89	7	Range: 79-91
Overall			86	7	n=10
Mass Transition m/z 246 to 169 (Proposed for Quantification)					
Liver	M510F53	0.01	97	3	Range: 92-100
		0.1	101	2	Range: 98-103
Overall			99	3	n=10
Mass Transition m/z 246 to 204 (Proposed for Confirmation)					
Liver	M510F53	0.01	108	5	Range: 98-113
		0.1	104	4	Range: 98-109
Overall			106	5	n=10

Table A 26: Characteristics for the analytical method used for independent laboratory validation of test method 1 for boscalid residues in animal matrices

	Boscalid
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ)
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points, matrix matched standards Calibration extending from 0.003 mg/kg to 0.20 mg/kg <u>Milk</u> r = 0.9999 (Quantification; m/z 343→307) r = 0.9986 (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends y = 9261 + 35522x (Quantification; m/z 343→307) y = 17453 + 913202x (confirmation; m/z 343→271) <u>Eggs</u> r = 0.9998 (Quantification; m/z 343→307) r = 0.9998 (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends y = 5644+362191x (Quantification; m/z 343→307) y = 10895+ 9130846x (confirmation; m/z 343→271) <u>Meat</u> r = 0.9985 (Quantification; m/z 343→307) r = 0.9989 (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends y = 829+ 282375x (Quantification; m/z 343→307) y = 10336+742875x (confirmation; m/z 343→271) <u>Fat</u> r = 0.9997 (Quantification; m/z 343→307) r = 0.9993 (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends y = 3987+431256x (Quantification; m/z 343→307) y = 19457 x + 1108622x (confirmation; m/z 343→271)

	Boscalid
Calibration range	0.150 ng/mL to 10 ng/mL (corresponds to 0.003 mg/kg to 0.2 mg/kg) Covering 30% LOQ to 20% highest fortification level
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in all matrices for the two (2) mass transitions
Stability of Analyte in Standard Solutions	Boscalid was found to be stable for at least 45 days when prepared in methanol and stored at frozen conditions (typically $\leq -18^{\circ}\text{C}$) in the dark
Stability of Analyte in Sample Extracts	Boscalid was found to be stable in final extracts of all animal matrices for at least 7 days when stored at typically 1°C to 10°C in the dark

Table A 27-1: Characteristics for the analytical method used for independent laboratory validation of test method 2 for boscalid residues in animal matrices

	Boscalid
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points, matrix matched standards Calibration extending from 0.003 mg/kg to 0.20 mg/kg <u>Liver</u> $r = 0.9996$ (Quantification; m/z 343→307) $r = 0.9996$ (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends $y = 8605 + 331510x$ (Quantification; m/z 343→307) $y = 21229 + 885155x$ (confirmation; m/z 343→271) <u>Kidney</u> $r = 0.9998$ (Quantification; m/z 343→307) $r = 0.9997$ (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends $y = 7767 + 365805x$ (Quantification; m/z 343→307) $y = 33158 + 982547x$ (confirmation; m/z 343→271)
Calibration range	0.150 ng/mL to 10 ng/mL (corresponds to 0.003 mg/kg to 0.2 mg/kg) Covering 30% LOQ to 20% highest fortification level
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in all matrices for the two (2) mass transitions
Stability of Analyte in Standard Solutions	Boscalid was found to be stable for 268 days when prepared in methanol and stored at typically 1°C to 10°C in the dark. Boscalid was found to be stable for at 8 days when prepared in acetonitrile/water (1/1, v/v) with 0.1 % formic acid and stored at typically 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	Boscalid was found to be stable in final extracts of all animal matrices for at least 7 days when stored at typically 1°C to 10°C in the dark.

Table A 27-2: Characteristics for the analytical method used for independent laboratory validation of test method 2 for M510F01 residues in animal matrices

	M510F01
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points, matrix matched standards Calibration extending from 0.003 mg/kg to 0.20 mg/kg <u>Liver</u> $r = 0.9992$ (Quantification; m/z 359→323) $r = 0.9989$ (confirmation; m/z 359→140) Regression residuals presented in residual plot with random distribution and no visible trends $y = 67139 + 7864766x$ (Quantification; m/z 359→323) $y = 25551 + 459838x$ (confirmation; m/z 359→140) <u>Kidney</u> $r = 0.9993$ (Quantification; m/z 343→307) $r = 0.9990$ (confirmation; m/z 343→271) Regression residuals presented in residual plot with random distribution and no visible trends $y = 38238 + 710593x$ (Quantification; m/z 343→307) $y = 12045 + 459813x$ (confirmation; m/z 343→271)
Calibration range	0.150 ng/mL to 10 ng/mL (corresponds to 0.003 mg/kg to 0.2 mg/kg) Covering 30% LOQ to 20% highest fortification level
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed; however, matrix matched standards were used for the calibration
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in all matrices for the two (2) mass transitions
Stability of Analyte in Standard Solutions	M510F01 was found to be stable for at least 718 days when prepared in methanol and stored at typically 1°C to 10°C in the dark. M510F01 was found to be stable for 8 days when prepared in acetonitrile/water (1/1, v/v) with 0.1 % formic acid and stored at typically 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	M510F01 was found to be stable in final extracts of all animal matrices for at least 7 days when stored at typically 1°C to 10°C in the dark.

Table A 28-1: Characteristics for the analytical method used for independent laboratory validation of test method 3 for M510F52 residues in animal matrices

	M510F52
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points, matrix matched standards Calibration extending from 0.003 mg/kg to 0.20 mg/kg <u>Liver</u> $r = 0.9996$ (Quantification; m/z 232→169) $r = 0.9991$ (confirmation; m/z 232→204) Regression residuals presented in residual plot with random

	M510F52
	distribution and no visible trends y = 12141+349426x (Quantification; m/z 232→169) y = 19916+254382x (confirmation; m/z 232→204)
Calibration range	0.150 ng/mL to 10 ng/mL (corresponds to 0.003 mg/kg to 0.2 mg/kg) Covering 30% LOQ to 20% highest fortification level
Assessment of matrix effects is presented	Yes Significant matrix effect ($\geq 20\%$) was observed, therefore matrix-matched standards were used
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in all matrices for the two (2) mass transitions
Stability of Analyte in Standard Solutions	M510F52 was found to be stable for at least 184 days when prepared in methanol and stored at typically 1°C to 10°C in the dark. M510F52 was found to be stable for 15 days when prepared in acetonitrile/water (1+1, v+v) with 0.1 % formic acid and stored at typically 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	M510F52 was found to be stable in final extracts of liver for at least 7 days when stored at typically 1°C to 10°C in the dark.

Table A 28-2: Characteristics for the analytical method used for independent laboratory validation of test method 3 for M510F53 residues in animal matrices

	M510F53
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 7 data points, matrix matched standards Calibration extending from 0.003 mg/kg to 0.20 mg/kg <u>Liver</u> r = 0.9996 (Quantification; m/z 246→169) r = 0.9996 (confirmation; m/z 246→204) Regression residuals presented in residual plot with random distribution and no visible trends y = 10379+431097x (Quantification; m/z 246→169) y = 10884+618244x (confirmation; m/z 246→204)
Calibration range	0.150 ng/mL to 10 ng/mL (corresponds to 0.003 mg/kg to 0.2 mg/kg) Covering 30% LOQ to 20% highest fortification level
Assessment of matrix effects is presented	Yes Significant matrix effect ($\geq 20\%$) was observed, therefore matrix-matched standards were used
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in all matrices for the two (2) mass transitions
Stability of Analyte in Standard Solutions	M510F53 was found to be stable for at least 184 days when prepared in methanol and stored at typically 1°C to 10°C in the dark. M510F53 was found to be stable for 7 days when prepared in acetonitrile/water (1/1, v/v) with 0.1 % formic acid and stored at typically 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	M510F53 was found to be stable in final extracts of liver for at least 7 days when stored at typically 1°C to 10°C in the dark.

Conclusion

Test methods 1, 2 and 3 were successfully independently validated for the determination of boscalid and its metabolites in animal matrices from the tested LOQ of 0.01 mg/kg according to the guidance

document(s) SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.
No addition or modification to the original method was done. Primary validation and independent laboratory validation were carried out at different locations and by different study personnel. No communication with the method developers or others familiar with the method was necessary to carry out the analysis. The analytical method meets the requirements of guidance document(s) SANTE/2020/12830, rev. 2.

A 2.1.2.2.2 Analytical method **2**

A 2.1.2.2.2.1 Method validation

Comments of zRMS:	The analytical method was successfully validated for the determination of boscalid in honey with LOQ of 0.01 mg/kg according to the guidance document SANTE/2020/12830, rev. 1.
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Reference: KCP 5.2/04 **05** (KCP 5.1.2/03)

Report Validation of the Analytical Method for the Determination of Residues of Boscalid in Honey, Report Amendment 1, Sahvorost, N., 2022, Study No. S22-00776, Sponsor Study No. UPL/2022/0354.

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Samples of honey were extracted with acetonitrile, after addition of water. Clean-up was carried out by partition into acetonitrile followed by dispersive SPE with PSA. Quantification was performed by use of LC-MS/MS detection.

Results and discussions

The method was successfully validated for the determination of boscalid in honey from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.

Table A 18: Recovery results from method validation of boscalid using the analytical method

Table A 16: Recovery Results from In-house Validation of Boscalid using the Analytical Method					
Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Honey	Boscalid	0.01	98	2	Range: 96-100
		0.1	101	1	Range: 100-102
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Honey	Boscalid	0.01	101	3	Range: 96-105
		0.1	102	1	Range: 101-104

Table A 19: Characteristics for the analytical method used for validation of boscalid residues in honey

	Boscalid
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ

	Boscalid
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 8 data points $r \geq 0.995$ Regression residuals presented in residual plot with random distribution and no visible trends
Calibration range	0.06 ng/mL to 6 ng/mL (corresponds to 0.003 mg/kg to 3 mg/kg) Covering 30% LIQ to 20% highest calibration level
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in honey for the two (2) mass transitions
Stability of Analyte in Standard Solutions	Boscalid was found to be stable for at least 12 days when prepared in acetonitrile and stored at typically 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	Boscalid was found to be stable in final extracts of honey for 9 days when stored at typically 1°C to 10°C in the dark.

Conclusion

The method was successfully validated for the determination of boscalid in honey from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation and monitoring purposes.

A 2.1.2.2.2.2 Independent laboratory validation

Comments of zRMS:	The primary method (Sahvorost, N., 2022) was independently validated for the determination of boscalid in honey with LOQ of 0.01 mg/kg according to the guidance document SANTE/2020/12830, rev. 1.
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Reference: KCP 5.2/02 **06**

Report Independent Laboratory Validation of the Analytical method for Determination of Residues of Boscalid in Honey, Rastogi, T., 2022, Study No. S22-00783, Sponsor Study No. UPL/2022/0845.

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The principle of the analytical procedure is in line with the primary method:

Homogenisation: Thoroughly stirring with spatula

Extraction: Acetonitrile and addition of water

Liquid/Liquid Partition: Adding content of Citrate-Kit-1 (Bekolut, CK-01-050)

Clean-up: Transferring an aliquot to PSA-Kit-01 (Bekolut, PK-01)

Dilution: DF 10 in methanol/water (1/1, v/v) containing 0.5% formic acid

Quantification: LC-MS/MS

Results and discussions

The method for the determination of boscalid in honey was independently validated according to the guidance document SANTE/2020/12830, rev. 1.

Table A 20: Recovery results from independent laboratory validation of boscalid using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Honey	Boscalid	0.01	102	3.3	Range: 99.3-108
		0.1	99.8	1.3	Range: 98.0-101
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Honey	Boscalid	0.01	99.6	2.6	Range: 98.5-104
		0.1	98.2	1.6	Range: 96.5-100

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

	Boscalid
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ)
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 8 data points $r \geq 0.99$ Regression residuals presented in residual plot with random distribution and no visible trends
Calibration range	0.06 ng/mL to 6 ng/mL (corresponds to 0.003 mg/kg to 3 mg/kg) Covering 30% LOQ to 20% highest calibration level
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed
Limit of determination/quantification	LOD: 0.003 mg/kg LOQ: 0.01 mg/kg in honey for the two (2) mass transitions
Stability of Analyte in Standard Solutions	The stability of the analyte in working solutions, e.g. stock, fortification and calibration solutions prepared in respective solvent is demonstrated in the method validation study.
Stability of Analyte in Sample Extracts	The stability of the analyte in the final extracts are demonstrated in the method validation study.

Conclusion

The method was successfully independently validated for the determination of boscalid in/on honey from the tested LOQ of 0.01 mg/kg according to the guidance document(s) SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.

No addition or modification to the original method other than optimisation of instrumental parameters was done. Primary validation and independent laboratory validation were carried out at different locations and by different study personnel. No communication with the method developers or others familiar with the method was necessary to carry out the analysis. The analytical method meets the requirements of guidance document(s) SANTE/2020/12830, rev. 2.

A 2.1.2.2.2.3 Confirmatory method

No confirmatory method is required (two mass transitions were monitored).

A 2.1.2.2.2.4 Extraction efficiency

Not applicable.

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

No new or additional studies have been submitted

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

A 2.1.2.4.1 Analytical method 1

A 2.1.2.4.1.1 Method validation

Comments of zRMS:

The analytical method was successfully validated for the determination of boscalid and its metabolites M510F47, M510F49 and M510F64 in/on groundwater and surface water in accordance to SANTE/2020/12830, rev.1 for monitoring. The limit of quantification (LOQ) was 0.10 µg/L for all the analytes in both water matrices.

According to SANTE/2020/12830, the analytical method was successfully validated at the above mentioned LOQ (0.10 µg/L) for surface water, then the validated analytical method would also cover the drinking water matrix and hence no separate validation in drinking water matrix was required.

Data for metabolites:

M510F49							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition : 323 m/z → 202 m/z (Quantification)							
Ground Water	0.10	95.3, 99.5, 108, 97.0, 104	101	5.2	5	91.5	12
	1.0	82.2, 85.7, 84.1, 79.2, 79.6	82.2	3.4	5		
Surface Water	0.10	110, 98.9, 114, 114, 105	108	5.9	5	94.4	16
	1.0	75.8, 86.9, 80.2, 80.6, 78.6	80.4	5.1	5		
Mass Transition : 323 m/z → 94 m/z (Confirmation)							
Ground Water	0.10	92.0, 96.7, 97.8, 95.1, 103	96.9	4.2	5	90.4	8.4
	1.0	83.5, 83.4, 86.3, 79.9, 86.1	83.8	3.1	5		
Surface Water	0.10	112, 102, 114, 108, 103	108	4.8	5	97.0	13
	1.0	80.5, 99.1, 83.2, 83.3, 85.0	86.2	8.6	5		

No residue above 30 % of the LOQ level was detected in any control sample final volume

Analyte: M510F49

Final determination as: M510F49

Residues calculated as: M510F49

M510F64							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicate s	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition : 155 m/z → 35 m/z (Quantification)							
Ground Water	0.10	105, 108, 99.3, 95.2, 108	103	5.5	5	97.9	6.8
	1.0	93.0, 91.3, 95.1, 91.8, 92.6	92.8	1.6	5		
Surface Water	0.10	103, 105, 100, 99.3, 111	104	4.5	5	101	4.9
	1.0	106, 97.4, 94.9, 98.7, 96.5	98.6	4.2	5		
Mass Transition : 157 m/z → 37 m/z (Confirmation)							
Ground Water	0.10	93.3, 96.6, 104, 92.2, 93.5	95.9	5.0	5	95.9	4.5
	1.0	101, 98.6, 92.4, 90.4, 96.7	95.9	4.6	5		
Surface Water	0.10	110, 115, 102, 93.2, 112	106	8.3	5	104	7.2
	1.0	110, 98.0, 98.7, 105, 96.4	102	5.6	5		

No residue above 30 % of the LOQ level was detected in any control sample final volume

Analyte: M510F64

Final determination as: M510F64

Residues calculated as: M510F64

M510F47							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition : 158 m/z → 122 m/z (Quantification)							
Ground Water	0.10	82.5, 92.5, 84.3, 93.8, 101	90.8	8.3	5	90.4	7.1
	1.0	99.9, 85.0, 86.0, 90.0, 89.1	90.0	6.5	5		
Surface Water	0.10	107, 106, 98.6, 111, 107	106	4.3	5	104	5.5
	1.0	108, 103, 104, 102, 90.9	102	6.3	5		
Mass Transition : 158 m/z → 94 m/z (Confirmation)							
Ground Water	0.10	90.8, 84.1, 82.4, 84.4, 94.6	87.3	6.0	5	89.0	6.3
	1.0	100, 88.7, 84.1, 92.5, 88.5	90.8	6.6	5		
Surface Water	0.10	109, 111, 108, 110, 111	110	1.3	5	105	5.1
	1.0	103, 103, 103, 103, 93.7	101	4.1	5		
No residue above 30 % of the LOQ level was detected in any control sample final volume							
Analyte: M510F47		Final determination as: M510F47		Residues calculated as: M510F47			
The method is acceptable.							

Reference:	KCP 5.2/03 07
Report	Validation of Analytical Method for Determination of Boscalid and its Metabolites M510F47, M510F49 and M510F64 in Ground Water and Surface Water, Rastogi, T., 2022, Study No. S22-00979, Sponsor Study No. UPL/2022/2227.
Guideline(s):	Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Before taking aliquots, the ground and surface water were shaken thoroughly and left to settled and particulates if present.

Stock solutions of the analytes were prepared by dissolving a weight of the test /reference items with the aid of an ultrasonic bath.

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

Matrix-matched calibration solutions were prepared using final sample volumes of control (untreated) samples of a respective matrix which were then fortified with solvent standard solutions.

All solutions were stored refrigerated (typically 1°C to 10°C) in brown glass vials in the dark.

The analytical method consists of LC-MS/MS detection with direct injection.

Chromatographic and Mass Spectrometric Conditions:

Chromatographic conditions:

HPLC system: PAL System HTS-xt autosampler, Agilent binary gradient HPLC pump Infinity II 1290, MayLab Mistra Switch column oven

Pre-column: Phenomenex C18 4x3mm)

Column Thermo Betasil C18 column: Length: 100 mm, i.d.: 2.1 mm, particle size: 5 µm)

Column oven temperature: 40 °C

Injection volume: 50 µL

Mobile phases:

Eluent A: Water + 0,5 % formic acid

Eluent B: Methanol + 0,5 % formic acid

Retention time: Boscalid: approx. 5.5 min

MS system: Applied Biosystems API TripleQuad 6500+System, SCIEX (Triple quadrupole mass spectrometer). Electrospray ionisation (ESI, Turbo Spray). Positive ion (for Boscalid), Multiple Reaction Monitoring (MRM). Mass transition monitored: 343→307 (quantitation) and 343→271 (confirmation)

Results and discussions

The objective of the study was to validate the analytical method for the determination of Boscalid and its Metabolites M510F47, M510F49 and M510F64 in/on groundwater and surface water in accordance to Guidance document SANTE/2020/12830, rev.1 for monitoring. The limit of quantification (LOQ) was 0.10 µg/L for all the analytes in both water matrices.

According to SANTE/2020/12830, the analytical method was successfully validated at the abovementioned LOQ (0.10 µg/L) for surface water, then the validated analytical method would also cover the drinking water matrix and hence no separate validation in drinking water matrix was required.

Table A 22: Recovery results from method validation of Boscalid using the analytical method

Matrix	Analyte	Fortification level (µg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Ground water	Boscalid	0.1	98.2	3.8	Range: 94.7-104
		1.0	95.1	1.3	Range: 93.5-96.6
Overall			96.6	3.2	
Surface water	Boscalid	0.1	97.6	5.1	Range: 92.5-104
		1.0	92.0	3.2	Range: 89.2-96.4
Overall			94.8	5.1	n=10
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Ground water	Boscalid	0.1	101	2.7	Range: 97.6-105
		1.0	94.3	2.1	Range: 92.4-97.2
Overall			97.5	4.1	n=10
Surface water	Boscalid	0.1	97.2	2.9	Range: 93.5-101
		1.0	91.1	2.8	Range: 88.2-93.3
Overall			94.2	4.4	n=10

Table A 23: Characteristics for the analytical method used for validation of boscalid residues in water

	Boscalid
Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30% of the LOQ.
Calibration (type, number of data points)	Matrix-matched calibration standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch.
Calibration range	Concentration range: 0.03 ng/mL to 2.0 ng/mL Corresponding mass fraction range: 0.03 µg/L to 2.0 µg/L Coverage: 30% of the LOQ to at least +20% of the highest analyte concentration level detected in a sample extract The validated range does not exceed two (2) orders of magnitude Quantification; 343m/z→307 m/z; $y=2.6e+006x+2.21e+004$ ($r=0.9999$) Confirmation; 343m/z→271m/z; $y=8.06e+005x+7.21e+003$ ($r=0.9998$)

	Boscalid
Assessment of matrix effects is presented	Yes Insignificant (<20%) for all analytes in both investigated water matrices.
Limit of determination/quantification	0.10 µg/L(lowest validated fortification level) 30% of the LOQ (lowest calibration standard)
Stability of Analytes in Standard Solutions	Within ± 10% for 30 days when prepared in methanol and stored at typically 1°C to 10°C in the dark.
Stability of Analyte(s) in Sample Extracts	Recoveries within 70%-120 % for all analytes in both investigated water matrices for at least 9 days when stored at typically 1°C to 10°C in the dark.

Conclusion

The analytical method by Rastogi 2022 (S22-00979) is acceptable for the determination of boscalid residues in water with LOQ of 0.10 µg/L. The method was found to be valid according to the guidance document SANTE/2020/12830, rev.1 for monitoring and complies with SANTE/2020/12830, rev.2

A 2.1.2.4.1.2 Independent laboratory validation

Comments of zRMS:	The analytical method of Rastogi, T., 2022 for the determination of boscalid and its metabolites M510F47, M510F49 and M510F64 in ground and drinking water was independently validated according to the guidance document SANTE/2020/12830, rev. 1 with LOQ of 0.10 µg/L for all the analytes in both water matrices.							
	Data for metabolites:							
	M510F49							
	Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
	Mass Transition m/z 323→202 (Used for Quantification)							
	Ground water	0.1	96, 100, 92, 99, 96	97	3	5	96	3
		1.0	98, 97, 96, 96 92	96	2	5		
	Drinking water	0.1	83, 89, 82, 87, 89	86	6	5	83	6
		1.0	84, 82, 78, 76, 77	79	4	5		
	Mass Transition m/z 323→94 (Used for Confirmation)							
Ground water	0.1	101, 108, 99, 104, 91	101	6	5	100	5	
	1.0	99, 99, 100, 101, 94	99	3	5			
Drinking water	0.1	83, 90 ,78, 91, 86	86	6	5	83	6	
	1.0	83, 77, 82, 78, 77	79	4	5			
No residue above 30 % of the LOQ level was detected in any control sample extract. Recoveries are without any blank correction.								
M510F64								
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	
Mass Transition m/z 155→35 (Used for Quantification)								
Ground water	0.1	90, 80, 82, 82, 83	83	5	5	86	5	
	1.0	93, 88, 89, 86, 90	89	3	5			
Drinking water	0.1	78, 78, 79, 84, 84	81	4	5	83	4	
	1.0	84, 87, 86, 84, 86	85	2	5			
Mass Transition m/z 157→37 (Used for Confirmation)								
Ground water	0.1	69, 80, 77, 78, 74	76	6	5	82	9	
	1.0	91, 90, 85 ,84, 87	87	3	5			
Drinking water	0.1	85, 75, 84, 82, 72	80	7	5	84	7	
	1.0	90, 86, 86, 89, 90	88	2	5			
No residue above 30 % of the LOQ level was detected in any control sample extract. Recoveries are without any blank correction.								

M510F47							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition m/z 158→122 (Used for Quantification)							
Ground water	0.1	100, 101, 104, 102, 101	102	1	5	102	1
	1.0	105, 103, 102, 102, 103	103	1	5		
Drinking water	0.1	105, 101, 102, 104, 105	103	2	5	104	1
	1.0	106, 104, 103, 104, 105	104	1	5		
Mass Transition m/z 158→94 (Used for Confirmation)							
Ground water	0.1	103, 102, 103, 101, 101	102	1	5	103	1
	1.0	105, 104, 103, 103, 104	104	1	5		
Drinking water	0.1	106, 103, 104, 104, 104	104	1	5	104	1
	1.0	106, 104, 103, 104, 105	104	1	5		

No residue above 30 % of the LOQ level was detected in any control sample extract.
Recoveries are without any blank correction.

No addition or modification to the original method other than optimisation of instrumental parameters was done. The ILV is acceptable.

Reference: KCP 5.2/04 08

Report Independent Laboratory Validation of an Analytical Method for Determination of Boscalid and its Metabolites M510F47, M510F49 and M510F64 in Ground and Drinking Water, Kaiser, M., 2023, Study No. S22-01028, Sponsor Study No. UPL/2022/21136.

Guideline(s): Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The principle of the analytical procedure is in line with the primary method:

Homogenisation: Shaker

Quantification: LC-MS/MS

Results and discussions

The method for the determination of boscalid in drinking water was independently validated according to the guidance document SANTE/2020/12830, rev. 1.

Table A 24: Recovery results from independent laboratory validation of boscalid using the analytical method

Matrix	Analyte	Fortification level (µg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Ground water	Boscalid	0.1	119	4	Range: 114-126
		1.0	119	2	Range: 116-122
Drinking water	Boscalid	0.1	106	3	Range: 103-111
		1.0	102	3	Range: 98-106
Mass Transition m/z 343 to 271 (Proposed for Confirmation)					
Ground water	Boscalid	0.1	117	4	Range: 112-121

Matrix	Analyte	Fortification level (µg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
		1.0	119	2	Range: 117-122
Drinking water	Boscalid	0.1	105	2	Range: 103-109
		1.0	104	3	Range: 100-107

Table A 25: Characteristics for the analytical method used for independent laboratory validation of boscalid residues in water

	Boscalid
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30 % LOQ
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 9 data points r = 0.9995 Regression residuals presented in residual plot with random distribution and no visible trends
Calibration range	Concentration range: 0.03 ng/mL to 2.0 ng/mL Corresponding mass fraction range: 0.03 µg/L to 2.0 µg/L Coverage: 30% of the LOQ to at least + 20% of the highest analyte concentration level detected in a sample extract
Assessment of matrix effects is presented	Yes No significant matrix effect (≥ 20%) was observed
Limit of determination/quantification	LOD: 0.03 µg/L LOQ: 0.1 µg/L

Conclusion

The method was successfully independently validated for the determination of boscalid and its metabolites M510F49, M510F64 and M510F47 in ground and drinking water from the tested LOQ of 0.1 µg/L up to 1.0 µg/L according to the guidance document SANTE/2020/12830, rev. 1 for monitoring. No addition or modification to the original method ther than optimisation of instrumental parameters was done. Primary validation and independent laboratory validation were carried out at different locations and by different study personnel No communication with the method developers or others familiar with the method was necessary to carry out the analysis and the first attempt made resulted in the validation data reported here.

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)

A 2.1.2.6.1 Analytical method 1

A 2.1.2.6.1.1 Method validation

Comments of zRMS:	The analytical method of Rastogi, T. (2022) has been successfully validated and fulfils the requirements of SANTE/2020/12830 for the analysis of boscalid and its metabolite M510F01 in/on body fluids (blood and urine) with LOQ of 0.01 mg/L for both analytes in both investigated matrices using LC-MS/MS. The method is acceptable.
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Reference:	KCP 5.2/05 09
Report	Development and Validation of an Multi-Residue Method QuEChERS for the Determination of Boscalid and its Metabolite M510F01 in Body Fluids, Rastogi T., 2022, Study No. S22-00982, Sponsor Study No. UPL/2022/2054.
Guideline(s):	Yes, SANTE/2020/12830, Rev.1 (February 24, 2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to validate an analytical method for the determination of boscalid and its metabolite M510F01 in/on body fluids (blood and urine) in accordance to guidance document SANTE/2020/12830, rev.1 for monitoring.

The limit of quantification was 0.01 mg/L for both analytes in both investigated matrices.

Samples of investigated matrices, after addition of water to bovine blood and human urine, were extracted with acetonitrile. The samples were shaken using a horizontal shaker. A SupelQuE Citrate Tube (55227-U) containing mixture of magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate and sodium citrate tribasic dehydrate was added. The extract was shaken and centrifuged. An aliquot of organic layer was transferred to another tube containing primary secondary amine (PSA, 55228-U). The samples were shaken and centrifuged. The samples were diluted in acetonitrile/water (1/1, v/v) containing 0.1% formic acid prior to the LC-MS/MS analysis for the determination of boscalid and M510F01.

Results and discussions

The method was successfully validated for the determination of boscalid and M510F01 in body fluids with LOQ of 0.01 mg/L according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.

Table A 26: Recovery results from method validation of boscalid and metabolite M510F01 using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 343 to 307 (Proposed for Quantification)					
Bovine Blood	Boscalid	0.01	112	2.0	Range: 109-115
		0.1	112	4.5	Range: 108-121
Human Urine	Boscalid	0.01	114	2.0	Range: 112-118
		0.1	116	2.0	Range: 115-120
Mass Transition m/z 343 to 140 (Proposed for Confirmation)					
Bovine Blood	Boscalid	0.01	116	4.2	Range: 109-120
		0.1	115	5.1	Range: 109-125
Human Urine	Boscalid	0.01	115	2.7	Range: 112-120
		0.1	115	2.8	Range: 111-119
Mass Transition m/z 359 to 323 (Proposed for Quantification)					
Bovine Blood	M510F01	0.01	107	3.9	Range: 102-112
		0.1	107	4.4	Range: 102-114
Human Urine	M510F01	0.01	109	5.8	Range: 104-119
		0.1	109	2.4	Range: 105-112

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 359 to 140 (Proposed for Confirmation)					
Bovine Blood	M510F01	0.01	109	2.8	Range: 105-113
		0.1	108	4.4	Range: 102- 116
Human Urine	M510F01	0.01	109	2.7	Range: 106 - 112
		0.1	109	2.9	Range: 106-113

Table A 27: Characteristics for the analytical method used for validation of boscalid and M510F01 residues in body fluids

	Boscalid	M510F01
Specificity	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ)	Mass spectrum is provided (2 mass transitions were evaluated) Blank value < 30% LOQ)
Calibration (type, number of data points)	Individual calibration data presented Calibration line equation presented 6 data points $r \geq 0.99$ Regression residuals presented in residual plot with random distribution and no visible trends	Individual calibration data presented Calibration line equation presented 6 data points $r \geq 0.99$ Regression residuals presented in residual plot with random distribution and no visible trends
Calibration range	Concentration range: 0.15 ng/mL to 10 ng/mL Corresponding mass fraction range: 0.003 mg/L to 0.20 mg/L Coverage: 30% of the LOQ to at least + 20% of the highest analyte concentration level detected in a sample extract	Concentration range: 0.15 ng/mL to 10 ng/mL Corresponding mass fraction range: 0.003 mg/L to 0.20 mg/L Coverage: 30% of the LOQ to at least + 20% of the highest analyte concentration level detected in a sample extract
Assessment of matrix effects is presented	Yes No significant matrix effect ($\geq 20\%$) was observed	Yes No significant matrix effect ($\geq 20\%$) was observed
Limit of determination/quantification	LOD: 30% of the LOQ (lowest calibration standard) LOQ: 0.01 mg/L	LOD: 30% of the LOQ (lowest calibration standard) LOQ: 0.01 mg/L
Stability of Analyte in Standard Solutions	The stability of the working solution (i.e. stock, fortification and intermediate calibration solutions) of boscalid in methanol was demonstrated for 30 days when stored at 1°C to 10°C in the dark in the study S22-00979.	The stability of the working solution (i.e. stock, fortification and intermediate calibration solutions) of M510F01 in methanol was demonstrated for 9 days when stored at 1°C to 10°C in the dark.
Stability of Analyte in Sample Extracts	Recoveries within 70% - 120% in all matrix extracts for 8 days when stored at typically 1°C to 10°C in the dark	Recoveries within 70% - 120% in all matrix extracts for 8 days when stored at typically 1°C to 10°C in the dark

Conclusion

The method was successfully validated for the determination of boscalid and M510F01 in body fluids with LOQ of 0.01 mg/L up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for monitoring. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation and monitoring purposes.

A 2.1.2.6.1.2 Confirmatory method

No confirmatory method is required (two mass transitions were monitored).

A 2.1.2.6.1.3 Extraction efficiency

Not applicable.

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

No new or additional studies have been submitted.

A 2.2 Analytical methods for Prothioconazole

A 2.2.1 Methods used for the generation of pre-authorization data (KCP 5.1)

Please refer to section A 2.1.1 for analytical methods used for the generation of pre-authorization data.

A 2.2.1.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.1)

A 2.2.1.1.1 Analytical method 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 5.1.2/10 (filed in KCP 5.2/06 10)

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

Please refer to point A 2.2.2.1 (reference KCP 5.2/06 10) for the study by Pearson, 2022, Report No. QG/20/011 that was used to generate pre-authorization data and is identical to the post-registration method.

A 2.2.1.1.2 Analytical method 2

A 2.2.1.1.2.1 Method validation

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 SANTE/2020/12830 rev. 1. The method is acceptable.
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Reference: KCP 5.1.2/11

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.

Residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole 6-hydroxy-desthio were extracted from oilseed rape seed, field beans (dried), barley grain, barley forage, cucumber and orange by shaking in acetonitrile: water (80:20 v/v) followed by filtration through Whatman filter paper.

Residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole 6-hydroxy-desthio were extracted from oilseed rape forage, wheat forage, wheat grain, barley straw and wheat straw with acetonitrile and QuEChERS extraction salts, followed by centrifugation. An aliquot of the acetonitrile phase was then taken and mixed with water.

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 μ m C18 100A 4.6 $\times$ 250 mm			
Guard Column:	C18 4 $\times$ 3.0 mm – Part no. AJ0-4287			
Column Oven Temperature (°C):	40			
Injection Volume * (μ L):	20			
Flow Rate (μ L/min):	1000			
Mobile Phase A:	0.1 % Formic acid in water			
Mobile Phase B:	Methanol			
Gradient:	Time (minutes)	Flow Rate (μ L/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 (CEP)	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 28: 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). $r \geq 0.995$

	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.
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.

A 2.2.1.1.2.2 Independent laboratory validation

Not relevant.

A 2.2.1.1.2.3 Confirmatory method (if required)

No confirmatory method is required.

A 2.2.1.1.2.4 Extraction efficiency

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 metabolism 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.

In the current study, residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole 6-hydroxy-desthio were extracted from oilseed rape seed, uses a similar extraction system (acetonitrile: water (80:20 v/v) and extraction efficiency is demonstrated.

A 2.2.1.1.3 Analytical method 3

A 2.2.1.1.3.1 Method validation

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 5.1.2/12

Report Triazole Derived Metabolites (TDM's) (1,2,4-Triazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops [D. Giancola, 2023](#) [B Phipps, R Toscano Saes, H Coleman, 2024](#) Report No. QG/20/012

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

Deviations: No

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 × 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 29: Characteristics for the analytical method used for validation of triazole derived metabolites residues in Oilseed Rape Plant

	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) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.0012 – 0.2 mg/kg) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.0240– 4.00 mg/kg) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.00240– 0.400 mg/kg) $r \geq 0.995$
Assessment of matrix effects is presented	Yes			
Limit of determination/quantification	LOQ = 0.005 mg/kg (Oilseed rapeseed, Oilseed rape plant) LOD = 0.0015 mg/kg (Oilseed rapeseed Oilseed rape plant)	LOQ = 00.005 mg/kg (Oilseed rapeseed, Oilseed rape plant) LOD = 0.0015 mg/kg (Oilseed rapeseed)	LOQ = 0.1 mg/kg (Oilseed rapeseed, Oilseed rape plant) LOD = 0.03 (Oilseed rapeseed, Oilseed rape plant)	LOQ = 0.01 mg/kg (Oilseed rapeseed, Oilseed rape plant) LOD = 0.003 (Oilseed rapeseed, Oilseed rape plant)

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.

A 2.2.1.1.3.2 Independent laboratory validation

Not relevant.

A 2.2.1.1.3.3 Confirmatory method (if required)

No confirmatory method is required.

A 2.2.1.1.3.4 Extraction efficiency

Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Draft Assessment Report DAR – PROTHIOCONAZOLE, July 2005, Volume 3, Annex B.5 and B7 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 metabolism 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.

A 2.2.1.1.4 Analytical method 4

A 2.2.1.1.4.1 Method validation

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, Lebrun F., 2024, Report No. 644-2023, Analytical phase code. S23-101384-L1 (TES-2307)
Guideline(s):	Yes - SANTE/2020/12830 rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method principle is based on 01062/M004 method as given in Prothioconazole_RAR_07_Volume_3CA_B-5_2018-02-22 (European Commission: Draft (Renewal) Assessment Report according to the Commission Regulation (EU) No 1107/2009: Prothioconazole, Volume 3 – B.5 (AS), February 2018) and is summarised below:

- Extraction with Methanol/water (4/1, v/v) of the homogenised honey
- Isotopically labelled standard: Isotopically labelled standards were added and used for quantification
- Final fill up: Evaporation to aqueous remainder (methanol free) and final fill up with water
- Storage: Final sample extracts were stored at 1 °C to 10 °C (target) in the dark until analysis
- Detection: Liquid chromatography coupled to differential ion mobility spectrometry and tandem mass spectrometry (LC-DMS-MS/MS)

For method validation in accordance to SANTE/2020/12830, rev. 2 at least the following types and numbers of samples were analysed over the course of the analytical phase.

- 2 control samples
 - 5 control samples fortified at the LOQ
 - 5 control samples fortified at 10x LOQ
- The analytes were fortified jointly and quantified separately.

Because two (2) mass transitions were not available for 1,2,4-triazole, triazole acetic acid and triazole lactic acid, two (2) independent LC-DMS-MS/MS detection methods were used, according to SANTE/2020/12830, rev. 2.

One of the two methods is proposed for quantification but both methods proved to be interchangeably applicable for quantification and confirmation.

Conversely, two (2) mass transitions were available for triazole alanine. One of the two mass transitions is proposed for quantification but both selected mass transitions were evaluated and proved to be interchangeably applicable for quantification and confirmation.

Recoveries for the validation were done at the same time as the analyses of the honey samples, therefore served also as procedural recoveries.

Instrument Conditions

Liquid Chromatography:

Detection method Nr.1

UPLC system	1290 Infinity Binary LC System, Agilent Technologies (UHPLC, ≤ 1200 bar)			
Pre-column	SecurityGuard™ ULTRA for C18 UHPLC (2.1 mm, Phenomenex, Art. No. AJO-8782)			
Column	Hypercarb (100 mm x 3 mm, 5 μ m, Thermo Scientific, Art. No. 35005-103030)			
Column oven temperature	60 °C			
Injection volume	20 μ L			
Mobile phases	Eluent A: Methanol containing 0.5 %formic acid (v/v) Eluent B: Water containing 0.5 %formic acid (v/v)			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [μ L/min]
	0.0	0	100	600
	3.0	0	100	600
	5.0	50	50	600
	8.0	50	50	600
	8.1	0	100	600
	11	0	100	600
Divert valve	to mass spectrometer from 1.0 min to 7.0 min			
Retention times (approx.)	1.5 min (1,2,4-Triazole) 5.8 min (Triazole Acetic Acid) 2.2 min (Triazole Alanine) 5.8 min (Triazole Lactic Acid)			

Detection method Nr.2

UPLC system	1290 Infinity Binary LC System, Agilent Technologies (UHPLC, ≤ 1200 bar)			
Pre-column	none			
Column	Aquasil C18 (150 mm x 3 mm, 3 µm, Thermo Scientific, Art. No. 77503-153030)			
Column oven temperature	60 °C			
Injection volume	20 µL			
Mobile phases	Eluent A: Methanol containing 0.5 %formic acid (v/v) Eluent B: Water containing 0.5 %formic acid (v/v)			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.00	5.0	95	600
	1.0	5.0	95	600
	1.3	40	60	600
	3.0	40	60	600
	3.1	95	5.0	600
	5.0	95	5.0	600
	5.1	5.0	95	600
	8.5	5.0	95	600
Divert valve	to mass spectrometer from 0.7 min to 3.5 min			
Retention times (approx.)	1.7 min (1,2,4-Triazole) 1.8 min (Triazole Acetic Acid) 1.8 min (Triazole Lactic Acid)			

Mass Spectrometry: Detection method Nr.2

MS system	TripleQuad 6500+ System, SCIEX (Triple quadrupole mass spectrometer with device for ion mobility spectrometry (SelexION))					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	5500 V	Ionspray turbo heater (TEM)		550 °C		
Curtain gas (CUR)	Nitrogen set at 35 (arbitrary units)	Gas flow 1 (GS1)		Zero-grade air set at 45 (arbitrary units)		
Collision gas (CAD)	Nitrogen set at 7 (arbitrary units)	Gas flow 2 (GS2)		Zero-grade air set at 65 (arbitrary units)		
Entrance Potential (EP)	10 V	DMS Temperature (DT)		Low		
Cell Exit Potential (CXP)	15 V	DMS Resolution Enhancement (DR)		Open		
Separation Voltage (SV)	3000 V	DMS Modifier (MD)		None		
Analyte Monitored	Mass Transition Monitored (m/z)	Time ^a [min]	Declustering Potential (DP) [V]	Collision Energy (CE) [V]	Compensation Voltage ^b (COV) [V]	DMS Offset (DMO) [V]
T	70 → 43	1.67	140	30	-14	25
T-IL-IS	75 → 46	1.67	140	30	-14	25
TAA	128 → 70	1.79	100	25	-4.5	15
TAA-IL-IS	133 → 75	1.79	100	25	-4.5	15
TLA	158 → 70	1.81	90	25	-0.5	15
TLA-IL-IS	163 → 75	1.81	90	25	-0.5	15

^a The retention times set for the scheduled MRM may need to be adjusted.

^b COV values may need to be adjusted on occasion to yield optimal selectivity and sensitivity. If results comparable to the results shown in this report cannot be obtained, SV values might also require adjustment.

Results and discussions

The analytical method was validated for the determination of residue in honey in compliance with the Guideline SANTE/2020/12830, Rev.2 of 14/02/2023 within this study at LOQ (0.01 mg/kg) and 10xLOQ (0.1 mg/kg) levels.

Table A 30: Recovery results from method validation of triazole derived metabolites residues using the analytical method

Honey							
Mass Transition	Fortification level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
1,2,4-Triazole							
m/z 70→43*	0.01	110, 101, 97, 104, 83	99	10	5	100	6.8
	0.1	102, 101, 100, 99, 99	100	1.3	5		
m/z 70→43#	0.01	99, 93, 91, 96, 105	97	6.0	5	95	4.6
	0.1	95, 92, 94, 96, 92	94	2.1	5		
TAA							
m/z 128→70*	0.01	98, 90, 90, 86, 94	92	4.7	5	94	4.2
	0.1	98, 98, 95, 97, 93	96	2.0	5		
m/z 128→70#	0.01	88, 90, 89, 94, 91	91	2.5	5	92	2.6
	0.1	95, 93, 94, 89, 92	93	2.3	5		
TA							
m/z 157→70*	0.01	92, 89, 92, 97, 95	93	3.4	5	94	2.8
	0.1	95, 98, 94, 94, 96	95	1.7	5		
m/z 157→88	0.01	89, 97, 93, 96, 101	96	4.7	5	94	3.7
	0.1	93, 95, 90, 93, 94	93	1.8	5		
TLA							
m/z 158→70*	0.01	93, 96, 98, 98, 89	95	4.1	5	97	4.0
	0.1	100, 103, 99, 99, 98	100	1.8	5		
m/z158→70#	0.01	94, 95, 97, 94, 97	95	1.9	5	95	1.7
	0.1	94, 94, 94, 92, 95	94	1.3	5		

Table A 31: Characteristics for the analytical method used for validation of triazole derived metabolites residues in honey

	TRZ	TAA	TA	TLA
Specificity	A second mass transition was not available. Therefore, a different and independent LC-DMS-MS/MS detection method was used to confirm the peak identities.	A second mass transition was not available. Therefore, a different and independent LC-DMS-MS/MS detection method was used to confirm the peak identities.	A second mass transition was monitored for confirmation of peak identity but was not used for the quantification of samples	A second mass transition was not available. Therefore, a different and independent LC-DMS-MS/MS detection method was used to confirm the peak identities.
	blank value < 30 % LOQ			
Calibration (type, number of data points)	Minimum of 8 calibration points. The calibration curves obtained for all analytes were linear since the regression residuals were randomly distributed. Furthermore, coefficients of determination (R^2) were ≥ 0.99 . Linear regression was performed with 1/x-weighting.			
	m/z 70/43 Q: $y=0.7115x+0.0029$ $r=0.9991$ m/z 70/43 C: $y=0.8284x+0.0020$ $r=0.9996$	m/z 128/70 Q: $y=0.9759x+0.0034$ $r=0.9995$ m/z 128/70 C: $y=1.2134x+0.0025$ $r=0.9996$	m/z 157/70: $y=1.0556x+0.0040$ $r=0.9996$ m/z 157/88: $y=0.4651x+0.0015$ $r=0.9993$	m/z 158/70 Q: $y=0.8493x+0.0022$ $r=0.9994$ m/z 158/70 C: $y=0.8101x+0.0013$ $r=0.9992$
Calibration range	0.30 ng/mL to 30 ng/mL (corresponds to a mass fraction level of 0.003 mg/kg to 0.3 mg/kg) and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any sample extract. The calibration curve did not exceed two (2) orders of magnitude.			

Assessment of matrix effects is presented	No, 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	LOQ: 0.01 mg/kg LOD: 0.003 mg/kg
Stability of Analytes in Standard Solutions	The stability of stock solutions diluted into water/methanol (4+1, v+v) was checked by the analytical laboratory (see Appendix B) and it was confirmed that stock solutions are stable when stored at 1 °C to 10 °C in the dark for at least 195 days. The stability of solution in final solvent (water) was checked by the analytical laboratory (see Appendix B) and it was confirmed that solutions in final solvent are stable when stored at 1 °C to 10 °C in the dark for at least 15 days
Stability of Analyte(s) in Sample Extracts	Since internal isotopically labelled standards were used for quantification and were added directly at the end of the sample extraction procedure, the stability of the analytes in sample extracts did not need to be investigated

Conclusion

Samples of honey were analysed for residues of 1,2,4-triazole (T), triazole acetic acid (TAA), triazole alanine (TA) and triazole lactic acid (TLA). Residues of all analytes were below the LOQ of 0.001 mg/kg.

The analytical method was validated for the determination of analytes in honey in compliance with the Guideline SANTE/2020/12830, Rev.2 of 14/02/2023 within this study at LOQ (0.01 mg/kg) and 10xLOQ (0.1 mg/kg) levels.

With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the samples of the study.

A 2.2.1.1.4.2 Independent laboratory validation

Not relevant.

A 2.2.1.1.4.3 Confirmatory method (if required)

No confirmatory method is required.

A 2.2.1.1.4.4 Extraction efficiency

Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Draft Assessment Report DAR – PROTHIOCONAZOLE, July 2005, Volume 3, Annex B.5 and B7 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 metabolism 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.

A 2.2.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 2.2.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

A 2.2.2.1.1 Analytical method 1

A 2.2.2.1.1.1 Method validation

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 5.2/06 10
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.

Results and discussions

The method was successfully validated for the determination of prothioconazole-desthio in crops from the tested LOQ of 0.005 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring.

Table A 32: Recovery results from method validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 312.1 to 70.1 (Proposed for Quantification)					
oilseed rape seed	prothioconazole-desthio	0.005	93	2.9	Range: 90-97
		0.05	3.0	2.1	Range: 87-95

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Overall			93	2.9	n=10
Mass Transition m/z 312.1 to 125.0 (Proposed for Confirmation)					
oilseed rape seed	prothioconazole-desthio	0.005	94	2.2	Range: 91-97
		0.05	93	2.7	Range: 89-96
Overall			93	2.4	n=10
Mass Transition m/z 312.1 to 70.1 (Proposed for Quantification)					
oilseed rape forage	prothioconazole-desthio	0.005	108	2.7	Range: 105-112
		0.05	110	1.7	Range: 106-111
Overall			109	2.3	n=10
Mass Transition m/z 312.1 to 125.0 (Proposed for Confirmation)					
oilseed rape forage	prothioconazole-desthio	0.005	108	3.9	Range: 105-115
		0.05	109	1.0	Range: 108-110
Overall			109	2.7	n=10

Table A 33: 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)	Individual calibration data presented Calibration line equation presented 8 data points r ≥ 0.995 Regression residuals presented in residual plot with random distribution and no visible trends
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 Covering 30% LOQ to 20% highest calibration level
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD: 0.0015 mg/kg LOQ = 0.005 mg/kg
Stability of Analyte in Standard Solutions	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 pre-prepared in acetonitrile:water (9:1, v/v) were shown to be stable for up to 71 days when stored refrigerated.
Stability of Analyte in Sample 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.

Conclusion

The method was successfully validated for the determination of prothioconazole-desthio in crops from the

tested LOQ of 0.005 mg/kg according to the guidance document SANTE/2020/12830, rev. 1 for risk assessment and/or monitoring. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation and monitoring purposes.

A 2.2.2.1.1.2 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 5.2/07 11
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 5.2/06). 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 34: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 312→70 (Quantification)					
Oilseed rape seeds	Prothioconazole-desthio	0.005	82	3	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Oilseed rape seeds	Prothioconazole-desthio	0.05	78	9	
Overall			80	7	n=10
Mass Transition m/z 312→125 (Confirmation)					
Oilseed rape seeds	Prothioconazole-desthio	0.005	82	4	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Oilseed rape seeds	Prothioconazole-desthio	0.05	79	9	
Overall			81	7	n=10

Table A 35: 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
Stability of Analyte in Standard Solutions	The stability of the analyte in working solutions, e.g. stock, fortification and calibration solutions prepared in respective solvent is demonstrated in the method validation study.
Stability of Analyte in Sample Extracts	The stability of the analyte in the final extracts are demonstrated in the method validation study.

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 2.2.2.1.1.3 Confirmatory method (if required)

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.1.1.4 Extraction efficiency

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 metabolism 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.

In the current study, residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole 6-hydroxy-desthio were extracted from oilseed rape seed, uses a similar extraction system (acetonitrile: water (80:20 v/v) and extraction efficiency is demonstrated.

A 2.2.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

A 2.2.2.2.1 Analytical method 1

A 2.2.2.2.1.1 Method validation

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 5.2/08 **12**

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	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 [#]	126	10	47	12	100
	312 → 125	126	10	49	6	100

[#] 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.

Table A 36: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
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 37: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in animal matrices

	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. The analytical method meets the requirements of guidance document(s) SANTE/2020/12830, rev. 2.

A 2.2.2.2.1.2 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 5.2/09 13
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 38: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

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

Table A 39: 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

	Prothioconazole-desthio
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). The analytical method meets the requirements of guidance document(s) SANTE/2020/12830, rev. 2.

A 2.2.2.2.1.3 Confirmatory method (if required)

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.2.1.4 Extraction efficiency

Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Draft Assessment Report DAR – PROTHIOCONAZOLE, July 2005, Volume 3, Annex B.5 and B7 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 metabolism 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.

A 2.2.2.2.2 Analytical method 2

A 2.2.2.2.2.1 Method validation

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 5.2/49 14
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 (February 24, 2021)
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 40: Recovery results from method validation of prothioconazole-desthio and hydroxy metabolites using the analytical method

Metabolites using the analytical method					
Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 312.1 to 70.1 (Proposed for Quantification)					
Honey	Prothioconazole-desthio	0.005	106	1.6	Range: 104-108
		0.05	107	2.1	Range: 104-109
Overall			106	1.8	n=10
Mass Transition m/z 312.1 to 125.0 (Proposed for Confirmation)					
Honey	Prothioconazole-desthio	0.005	106	1.0	Range: 106-108
		0.05	105	2.0	Range: 104-109
Overall			106	1.5	n=10
Mass Transition m/z 328.265 to 69.900 (Proposed for Quantification)					
Honey	Prothioconazole- α -hydroxy-desthio	0.005	85	1.6	Range: 83-86
		0.05	84	10	Range: 71-95
Overall			84	8.1	n=10
Mass Transition m/z 328.265 to 141.200 (Proposed for Confirmation)					
Honey	Prothioconazole- α -hydroxy-desthio	0.005	86	4.0	Range: 83-86
		0.05	87	2.3	Range: 71-95
Overall			87	3.2	n=10
Mass Transition m/z 328.142 to 70.000 (Proposed for Quantification)					
Honey	Prothioconazole-3-hydroxy-desthio	0.005	89	0.6	Range: 88-90
		0.05	86	9.5	Range: 72-96
Overall			87	7.7	n=10
Mass Transition m/z 328.142 to 141.000 (Proposed for Confirmation)					
Honey	Prothioconazole-3-hydroxy-desthio	0.005	88	1.7	Range: 86-90
		0.05	86	8.4	Range: 74-95
Overall			87	6.8	n=10

Mass Transition m/z 328.154 to 70.000 (Proposed for Quantification)					
Honey	Prothioconazole-4-hydroxy-desthio	0.005	80	1.6	Range: 78-81
		0.05	83	2.9	Range: 81-86
Overall			91	3.2	<i>n=10</i>
Mass Transition m/z 328.154 to 141.000 (Proposed for Confirmation)					
Honey	Prothioconazole-4-hydroxy-desthio	0.005	80	1.1	Range: 79-81
		0.05	83	2.5	Range: 81-86
Overall			81	3.1	<i>n=10</i>
Mass Transition m/z 327.948 to 70.000 (Proposed for Quantification)					
Honey	Prothioconazole-5-hydroxy-desthio	0.005	88	1.4	Range: 86-89
		0.05	87	10	Range: 71-97
Overall			87	8.3	<i>n=10</i>
Mass Transition m/z 327.948 to 141.000 (Proposed for Confirmation)					
Honey	Prothioconazole-5-hydroxy-desthio	0.005	87	1.6	Range: 86-89
		0.05	90	2.8	Range: 88-94
Overall			88	2.6	<i>n=10</i>
Mass Transition m/z 327.899 to 140.872 (Proposed for Quantification)					
Honey	Prothioconazole-6-hydroxy-desthio	0.005	88	9.7	Range: 79-95
		0.05	78	2.4	Range: 76-80
Overall			84	9.4	<i>n=10</i>
Mass Transition m/z 327.899 to 69.824 (Proposed for Confirmation)					
Honey	Prothioconazole-6-hydroxy-desthio	0.005	89	10.9	Range: 78-100
		0.05	78	2.8	Range: 76-81
Overall			84	10.4	<i>n=10</i>

Table A 41: 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 No significant matrix effect (≥ 20%) was observed	
Limit of determination/quantification	LOD = 0.00125 mg/kg LOQ = 0.005 mg/kg	LOD = 0.0015 mg/kg LOQ = 0.005 mg/kg
Stability of Analyte in Standard Solutions	Assessed and found to be stable: Standard solutions of prothioconazole-desthio (5) hydroxy prepared in acetonitrile:water (80:20, v/v) were shown to be stable for up to 54 days when stored refrigerated. Standard solutions of prothioconazole-desthio prepared in acetonitrile:water (80:20, v/v) were shown to be stable for up to 16 days when stored refrigerated.	

	Prothioconazole-desthio	Prothioconazole-desthio hydroxy metabolites
Stability of Analyte in Sample Extracts	Assessed and found to be stable: Prothioconazole-desthio was shown to be stable in honey sample extracts when stored refrigerated for at least 3 days. Prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5 hydroxy-desthio, prothioconazole-6-hydroxy-desthio and prothioconazole- α -hydroxy-desthio were shown to be stable in honey sample extracts when stored refrigerated for at least 12 days.	

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. The data meet the acceptance criteria of SANTE/2020/12830, Rev.2 for support of risk assessment data generation and monitoring purposes.

A 2.2.2.2.2 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 5.2/11 15
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 42: 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
Mass Transition m/z 312 to 70 (Proposed for Quantification)					
Honey	prothioconazole-desthio	0.005	105	2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
		0.05	110	2	
Overall			108	3	n=10
Mass Transition m/z 312 to 125 (Proposed for Confirmation)					
Honey	prothioconazole-	0.01	108	4	All mean recoveries were

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Mass Transition m/z 312 to 70 (Proposed for Quantification)					
	desthio	0.1	111	2	within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Overall			109	3	n=10

Table A 43: Characteristics for the analytical method used for independent laboratory validation of boscalid 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 No significant matrix effect (≥ 20%) was observed
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. The analytical method meets the requirements of guidance document(s) SANTE/2020/12830, rev. 2.

A 2.2.2.2.3 Confirmatory method

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.2.4 Extraction efficiency

Not applicable.

A 2.2.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

A 2.2.2.3.1 Analytical method 1

A 2.2.2.3.1.1 Method validation

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 5.2/42 **16**

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-desthio. The LOD was defined as the lowest calibration standard, corresponding to 0.002 mg/kg for prothioconazole and 0.0006 mg/kg for prothioconazole-desthio, which is 30 % of the LOQ.

Table A 44: Recovery results from method validation of prothioconazole and prothioconazole-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				
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 45: 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 ≥ 0.99	0.12 - 12 ng/mL, equivalent to 0.006 - 0.6 mg/kg r ≥ 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 2.2.2.3.1.2 Independent laboratory validation

Not relevant.

A 2.2.2.3.1.3 Confirmatory method (if required)

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.3.1.4 Extraction efficiency

Not applicable.

A 2.2.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

A 2.2.2.4.1 Analytical method 1

A 2.2.2.4.1.1 Method validation

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
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	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 5.2/13 17
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 (R²) 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 $0.05\ \mu\text{g/L}$ was confirmed for prothioconazole and prothioconazole-desthio in drinking and surface water.

Table A 46: Recovery results from method validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level (~~mg/kg~~ μg/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 47: 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	$0.0135 - 1.35\ \text{ng/mL}$, equivalent to $0.0149 - 1.49\ \mu\text{g/L}$ $r \geq 0.99$	
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOQ = $0.05\ \mu\text{g/L}$ LOD = $0.0149\ \mu\text{g/L}$	

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.

A 2.2.2.4.1.2 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 5.2/14 18
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 48: 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
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 49: 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 ≥ 0.999
Assessment of matrix effects is presented	yes

	Prothioconazole and Prothioconazole-desthio
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 2.2.2.4.1.3 Confirmatory method (if required)

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.4.1.4 Extraction efficiency

Not applicable.

A 2.2.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

A 2.2.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

A 2.2.2.6.1 Analytical method 1

A 2.2.2.6.1.1 Method validation

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 5.2/45 19

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	Declustering 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 50: 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 51: 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	
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.

A 2.2.2.6.1.2 Independent laboratory validation

Not relevant.

A 2.2.2.6.1.3 Confirmatory method (if required)

No confirmatory method is required (two mass transitions were monitored).

A 2.2.2.6.1.4 Extraction efficiency

Not applicable.

A 2.2.2.7 ~~A.2.A.9~~ Other Studies/ Information

No new or additional studies have been submitted.