

FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: BSK-FUN 500 SC

Product name(s): -

Chemical active substance:

boscalid, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant:

Pestila Sp. z o. o. and ProAgri International Sp. z o. o.

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Version history

When	What
April 2024	Submission date
August 2024	Additional data provided by the Applicant
10.2024	zRMS evaluation
02.2025	The final Registration Report.
06.2025	Corrections made after the commenting period.

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5 Analytical methods

5.1 Conclusion and summary of assessment

Analytical methods for the determination of residues

Data gaps:

- According to MRL Reg. (EU) 2022/1324, the MRL for muscle has been set at 0.01 mg/kg. LOQ of the EU agreed analytical methods for muscle is 0.025 mg/kg.

New methods for muscle (primary and ILV) with LOQ at 0.01 mg/kg are required (data gap). This gap can be filled after registration (within two years).

- The LOQ of the method of Class, 2000 for eggs, is not sufficient for currently stated MRL.

The method of Kampke Thiel, 2001 is not validated for eggs and meat.

Data gap: ILV method for eggs. This gap can be filled after registration (within two years).

- ILV method for drinking water should be provided after renewal of the active substance.

- Analytical methods for determination of boscalid residues in body fluids and tissues should be provided after renewal of the active substance.

These data gaps are anticipated to be addressed at active substance level in context with the renewal.

Commodity/crop	Supported/ Not supported
Cereals	Supported
Winter 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 in plant protection product is provided as follows:

Comments of zRMS:	Analytical method (HPLC/UV/DAD) for determination of active substances Boscalid in plant protection product BSK-FUN 500 SC has been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable.
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Reference: 5.1.1/01
5.1.1/02

Report BSK-FUN 500 SC: Validation of the Analytical Method for the Determination of the Active Ingredient Content. Garofani S., 2023, report no. CH-0853-2023

Guideline(s): Yes, SANCO/3030/99 rev.5 (22/03/19)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Determination of boscalid in BSK-FUN 500 SC was performed with HPLC using an internal standard and UV detector.

Equipment and chromatographic conditions for boscalid analysis

- High performance liquid chromatograph equipped with UV/Vis or DAD detector,
- Quaternary pump, auto sampler and software for instrument management and data reprocessing;
- Analytical balance, 0.1 mg precision;
- Freezer;
- Refrigerator;
- Ultrasonic bath;
- Volumetric glassware: pipettes, flasks, measuring cylinders;
- Plastic syringe without needle;
- Syringe filter 25 mm PTFE 0.45 µm;
- Usual laboratory glassware.

Under these chromatographic conditions, the retention time was 11.3 ± 0.1 min.
The total analysis time was 20 min.

Chromatographic condition:

HPLC column	Phenomenex or equivalent: Luna C18 100 Å, 5 µm, 250 x 4.6 mm i.d.
Detector	UV/Vis operating at 278 nm from 0 to 10 minutes UV/Vis operating at 240 nm from 10 to 25 minutes
Column temperature	25°C
Eluent A	Water
Eluent C	Acetonitrile
Eluent D	H ₃ PO ₄ 0.1 % v/v solution
Eluent (isocratic)	A / C / D = 20 / 70 / 10 % v/v/v
Eluent flow	1.0 mL/min
Volume of injection	10 µL
Boscalid ret. time	about 5.8 minutes
Dibutyl phthalate ret. time	about 15.7 minutes
Total analysis time	25 minutes

The preparation of standard solution

Using an analytical balance and a volumetric flask, prepare a stock reference material solution as follows:

Reference Material or Internal Standard	Nominal weight (mg)	Volume (mL)	Solvent	Dilution	Solvent
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Boscalid	10	10	Acetonitrile	1:20	Acetonitrile
Dibutyl phthalate	10				

Store the standard solutions in a refrigerator.

The preparation of specimen solutions

Using the analytical balance, weigh about 584.3 mg of the test item and about 250 mg of internal standard into a 50 mL conical flask, add 50 mL of acetonitrile.

Place into an ultrasonic bath for 5 minutes, filter using syringe filter at 0.45 µm, then dilute 1:100 with acetonitrile and transfer an aliquot into a vial for the HPLC analysis.

The summary of test item preparation procedure is presented in the table here below:

	Nominal weight (mg)	Volume (mL)	Solvent	Dilution	Solvent
Test item	584.3	50	Acetonitrile	1:100	Acetonitrile
Internal standard	250				

Store the test item solutions in a refrigerator.

Preparation of the fortified placebo solutions

Spike code	Placebo (mg)	Test substance (mg)	Internal standard weight (mg)	Solvent	Volume (mL)
Spike A	344.9	252.4	264.8	Acetonitrile	50
Spike B	347.5	248.8	249.4		50
	Dilution		Solvent		
Spike A	1:100		Acetonitrile		
Spike B	1:100		Acetonitrile		

The fortified samples were analysed by HPLC/UV.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance boscalid in plant protection product BSK-FUN 500 SC

	Boscalid
Author(s), year	Garofani S., 2023
Principle of method	SANCO/3030/99 rev.5, 22 March 2019
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of the analytical method was assessed using five boscalid standard solutions in the concentration range from 0.5219 mg/mL to 1.2177 mg/mL 29.22 – 68.19 µg/ml (261 – 609 g/L*). $y=1.12744x+0.02921$ Correlation coefficient: $R^2 > 0.99$ Required: $R^2 \geq 0.99$.
Precision – Repeatability Mean n = 5 (%RSD)	43.6% w/w RSDr = 1.52 RSD % = 0.15 Horrat value: 0.10
Accuracy n = 2 (% Total Recovery)	Palcebo Spiked A: 414.37 g/kg: 101.96% Palcebo Spiked B: 409.15 g/kg: 101.43% Mean (n=2) 101.7% (range: 101.43% – 101.96%)

	Boscalid
	Required: 97% - 103%
Interference/ Specificity	Fulfilled.
Comment	No comments.

*Calculated with respect to the nominal test item weight, considering the 1.1668 g/mL density value

Conclusion.

The HPLC method with HPLC using an internal standard and UV detector, used to quantify boscalid in BSK-FUN 500 SC was fully validated. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

Not relevant. BSK-FUN 500 SC does not contain relevant impurities.

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

Not relevant. BSK-FUN 500 SC does not contain materials of toxicological, ecotoxicological or environmental concern.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

A CIPAC analytical method for the determination of boscalid [673] in TC, WG, SC and SE formulations by RP-HPLC exists in CIPAC Handbook N.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of boscalid for the generation of pre-authorization data is given in the following table. For the detailed evaluation of new studies it is referred to Appendix 1.

Table 5.2-2: 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
<u>Elendt M7 medium</u> <i>Daphnia</i> sp., Acute Immobilisation Test (Ecotoxicology)	Primary & confirmatory	10 µg/L	UHPLC-MS/MS	Kolek L., 2024, report no. ETOX-2023-20
<u>AAP medium</u> Freshwater Alga and	Primary & confirmatory	1 µg/L	UHPLC-MS/MS	Kolek L., 2024, report no. ETOX-2023-21

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
Cyanobacteria, Growth Inhibition Test (Ecotoxicology)				
<u>0.1% Triton X-100 solution</u> Bumblebees (<i>Bombus</i> spp.), Acute Contact Toxicity Test (Ecotoxicology)	Primary	2 mg/L (nominal concentration) 1 mg/L (measured concentration)	UHPLC-DAD	Szlauer,S., 2023, report no. ETOX-2023-23
<u>50% Sucrose Syrup</u> Bumblebees (<i>Bombus</i> spp.), Acute Oral Toxicity Test (Ecotoxicology)	Primary	2 mg/L (nominal concentration) 1 mg/L (measured concentration)	UHPLC-DAD	Szlauer,S., 2023, report no. ETOX-2023-22
<u>Water</u> Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test (Ecotoxicology)	Primary	2 mg/L (nominal concentration) 1 mg/L (measured concentration)	UHPLC-DAD	Wesołowska K., 2024, report no. ETOX-2023-28
<u>Water</u> Terrestrial Plant Test: Vegetative Vigour Test (Ecotoxicology)	Primary	2 mg/L (nominal concentration) 1 mg/L (measured concentration)	UHPLC-DAD	Wesołowska K., 2024, report no. ETOX-2023-29
<u>Sucrose solution</u> Honeybees (<i>Apis mellifera</i> L.), Chronic Oral Toxicity Test (Ecotoxicology)	Primary & confirmatory	52.330 mg/kg (122.04 mg/kg referred as test item)	UHPLC-TOF-MS/MS	Mautino G., 2024, report no. 1142.1F.SAG23
<u>Water</u> Honeybees (<i>Apis mellifera</i> L.), Larval Toxicity Test Following Repeated Exposure (Ecotoxicology)	Primary & confirmatory	0.5233 g/L	UHPLC-TOF-MS/MS	Mautino G., 2024, report no. 1143.1F.SAG23
<u>Wheat (seeds, whole plant, straw</u> Magnitude of residues (Residues)	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Sala A., 2023, report no. LBN-0118-2023
<u>Rape (seeds, whole plant, plant without pods, pods)</u> Magnitude of residues (Residues)	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Sala A., 2023, report no. LBN-0119-2023
<u>Artificial soil</u> Earthworm Reproduction Test (<i>Eisenia andrei</i>) (Ecotoxicology)	Primary & confirmatory	43 µg/kg	UHPLC-MS/MS	Wesołowska K., 2024, report no. ETOX-2023-26
<u>Honey</u> Magnitude of residues	Primary & confirmatory	0.01 mg/kg	LC-MS/MS	Schlewitz P., 2023, report no. R C3128

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
(Residues)				

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

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

5.3.2 Description of analytical methods for the determination of residues 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	Reg. (EU) 2022/1324
Plant, high acid content		0.01 mg/kg	Reg. (EU) 2022/1324
Plant, high protein/high starch content (dry commodities)		0.15 mg/kg	Reg. (EU) 2022/1324
Plant, high oil content		0.01 mg/kg	Reg. (EU) 2022/1324
Plant, difficult matrices (hops, spices, tea)		0.4 mg/kg	Reg. (EU) 2022/1324
Muscle	boscalid	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		0.07 mg/kg	Reg. (EU) 2022/1324
Liver, kidney	Sum of boscalid and its hydroxy metabolites 2-chloro-N-(4'-chloro-5-hydroxybiphenyl-2-yl) nicotinamide (free and conjugated) expressed as	0.05 mg/kg	Reg. (EU) 2022/1324

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
	boscalid		
Soil (Ecotoxicology)	boscalid	0.05 mg/kg	common limit
Drinking water (Human toxicology)	boscalid, M510F47, M510F49	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	boscalid, M510F47, M510F49	125 µg/L	Lowest NOEC from aquatic toxicity study on <i>O. mykiss</i>
Air	boscalid	30 µg/m ³ (AOEL systemic of 0.1 mg/kg bw/d)	Calculated according to SANTE/2020/12830, Rev.2 14. February 2021
Tissue (meat or liver)	boscalid	0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023
Body fluids		0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023

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. No new studies have been submitted with this application.

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 0.02 mg/kg 0.05 mg/kg	GC-MS HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2001)
	ILV	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
	Confirmatory (if required)	0.01 mg/kg 0.05 mg/kg	GC-MS HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2001)
High acid content	Primary	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
	ILV	0.02 mg/kg 0.05 mg/kg	GC-MS HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2001)
	Confirmatory (if required)	0.02 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
High oil content	Primary	0.01 mg/kg	GC-MS	DAR 2002 (Weeren and Pelz,

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
				1999)
	ILV	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
	Confirmatory (if required)	0.05 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
High protein/high starch content (dry)	Primary	0.03 mg/kg 0.04 mg/kg 0.05 mg/kg	GC-MS HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2001)
	ILV	-	-	-
	Confirmatory (if required)	0.02 mg/kg 0.05 mg/kg	GC-MS HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2001)
Difficult (if required, depends on intended use)	Primary	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
	ILV	-	-	-
	Confirmatory (if required)	-	-	-

Some of the methods from table above showed higher LOQs than the required MRL of 0.01 mg/kg (high water content, high acid content and high oil content matrices: Funk & Mackenroth, 2000 for and Weeren & Pelz, 1999 as well as for high oil content: Reichert, 2001).

However, EFSA Journal 2014;12(7):3799 stated:

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.

No additional method is required.

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	DAR, 2002 (Bross M., 2001)
Not required, because:	-

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. No new studies have been submitted with this application.

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

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
Milk	Primary	0.01 mg/kg	GC-ECD HPLC-MS/MS	DAR 2002 (Class, 2000) DAR 2002 (Grosshans, 2000)
	ILV	0.01 mg/kg	GC-ECD	DAR 2002 (Kampke-Thiel, 2001)
	Confirmatory (if required)	0.01 mg/kg	GC-MS	DAR 2002 (Class, 2000)
Eggs	Primary	0.025 mg/kg 0.01 mg/kg	GC-ECD HPLC-MS/MS	DAR 2002 (Class, 2000) DAR 2002 (Grosshans, 2000)
	ILV	0.01 mg/kg 0.025 mg/kg	GC-ECD	DAR 2002 (Kampke-Thiel, 2001)
	Confirmatory (if required)	0.025 mg/kg	GC-MS	DAR 2002 (Class, 2000)
Muscle	Primary	0.025 mg/kg	GC-ECD HPLC-MS/MS	DAR 2002 (Class, 2000) DAR 2002 (Grosshans, 2000)
	ILV	0.025 mg/kg	GC-ECD	DAR 2002 (Kampke-Thiel, 2001)
	Confirmatory (if required)	0.025 mg/kg	GC-MS	DAR 2002 (Class, 2000)
Fat	Primary	0.025 mg/kg	GC-ECD HPLC-MS/MS	DAR 2002 (Class, 2000) DAR 2002 (Grosshans, 2000)
	ILV	0.025 mg/kg	GC-ECD	DAR 2002 (Kampke-Thiel, 2001)
	Confirmatory (if required)	0.025 mg/kg	GC-MS	DAR 2002 (Class, 2000)
Honey	Primary & confirmatory	0.01 mg/kg	LC-MS/MS	Schlewitz P., 2023, report no. R C3128
Component of residue definition: Sum of boscalid and its hydroxy metabolite 2-chloro-N-(4'-chloro-5-hydroxybiphenyl-2-yl) nicotinamide (free and conjugated) expressed as boscalid				
Kidney, liver	Primary	0.025 mg/kg	GC-ECD HPLC-MS/MS	DAR 2002 (Class, 2000) DAR 2002 (Grosshans, 2000)
	ILV	0.025 mg/kg	GC-ECD	DAR 2002 (Kampke-Thiel, 2001)
	Confirmatory (if required)	0.025 mg/kg	GC-MS	DAR 2002 (Class, 2000)

zRMS:

- According to MRL Reg. (EU) 2022/1324, the MRL for muscle has been set at 0.01 mg/kg. LOQ of the EU agreed analytical methods for muscle is 0.025 mg/kg.
New methods for muscle (primary and ILV) with LOQ at 0.01 mg/kg are required (data gap). This gap can be filled after registration (within two years).
- The LOQ of the method of Class, 2000 for eggs, is not sufficient for currently stated MRL. The method of Kampke Thiel, 2001 is not validated for eggs and meat.
Data gap: ILV method for eggs. This gap can be filled after registration (within two years).

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	-
Not required, because:	No residues > LOQ are expected.

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. No new studies have been submitted with this application.

Table 5.3-6: Validated methods for soil

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	DAR 2002 (Keller, 1998a)
Confirmatory	-	-	-

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. No new studies have been submitted with this application.

Table 5.3-7: Validated methods for water

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	Primary	0.05 mg/kg µg/L	GC-MS	DAR 2002 (Keller, 1998b)
	ILV	Not available in DAR. This data gap is anticipated to be addressed at active substance level in context with the renewal.		
	Confirmatory	-	-	-
Surface water	Primary	0.5 mg/kg µg/L	GC-MS	DAR 2002 (Grote, 2001)
	Confirmatory	-	-	-

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. No new studies have been submitted with this application.

Table 5.3-8: 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	DAR 2002 (Zangmeister, 2000)
Confirmatory	-	-	-

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

Since boscalid is not classified as toxic or highly toxic, in DAR for boscalid, 2002 (incl. Addendum 1, 2006) methods for body fluids and tissues are not required and are not available. However, according to the Regulation No. 283/2013 and to the SANTE/2020/12830, Rev.2, 14. February 2023 an analytical method for the determination of residues in body fluids and tissues for enforcement/monitoring purposes is required.

In Applicant opinion no additional studies are necessary and this data gap should be addressed at active substance level in context with the renewal of boscalid.

5.3.2.8 Other studies/ information

No new or additional studies have been submitted

Appendix 1 Lists of data considered in support of the evaluation

Tables considered not relevant can be deleted as appropriate.

MS to blacken authors of vertebrate studies in the version made available to third parties/public.

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	Garofani S.	2023	BSK-FUN 500 SC: Validation of the Analytical Method for the Determination of the Active Ingredient Content report No: CH-0853-2023 ChemService S.r.l. Controlli e Ricerche GLP Studies Department GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/01 (filled as KCP 10.2.1.2/01)	Kolek L.	2024	<i>Daphnia</i> sp., Acute Immobilisation Test report no. ETOX-2023-20 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**
			Amendment No. 1 <i>Daphnia</i> sp., Acute Immobilisation Test report no. ETOX-2023-20 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No		
KCP 5.1.2/02 (filled as KCP 10.2.1.3/01)	Kolek L.	2024	Freshwater Alga and Cyanobacteria, Growth Inhibition Test report no. ETOX-2023-21 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**

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
			Amendment No. 1 Freshwater Alga and Cyanobacteria, Growth Inhibition Test report no. ETOX-2023-21 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No		
KCP 5.1.2/03 (filled as KCP 10.3.1.1.2/02)	Szlauer S.	2023	Bumblebees (<i>Bombus spp.</i>), Acute Contact Toxicity Test report no. ETOX-2023-23 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/04 (filled as KCP 10.3.1.1.1/02)	Szlauer S.	2023	Bumblebees (<i>Bombus spp.</i>), Acute Oral Toxicity Test report no. ETOX-2023-22 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/05 (filled as KCP 10.6.2/01)	Wesołowska K.	2024	Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test report no. ETOX-2023-28 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/06 (filled as KCP 10.6.2/02)	Wesołowska K.	2024	Terrestrial Plant Test: Vegetative Vigour Test report no. ETOX-2023-29 EcoTox Alliance Sp. z o. o. GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/07 (filled as KCP 10.3.1.2/01)	Mautino G.	2024	Effects of BSK-FUN 500 SC on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Chronic Oral Toxicity Test report no. 1142.1F.SAG23 SAGEA Centro di Saggio s.r.l. GLP: Yes	N	Pestila* ProAgri**

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
			Published: No		
KCP 5.1.2/08 (filled as KCP 10.3.1.4/01)	Mautino G.	2024	Effects of BSK-FUN 500 SC on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure report no. 1143.1F.SAG23 SAGEA Centro di Saggio s.r.l. GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/09	Sala A.	2023	Magnitude of the residue of Boscalid (188425-85-6) in wheat (Raw Agricultural Commodity – RAC) grown in open field conditions after one application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe report no. LBN-0118-2023 LabAnalysis s.r.l. GLP: Yes Published: No	N	Pestila* ProAgri**
	Sala A.	2024	Final Report Amendment 1 Magnitude of the residue of Boscalid (188425-85-6) in wheat (Raw Agricultural Commodity – RAC) grown in open field conditions after one application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe report no. LBN-0118-2023 LabAnalysis Life Science s.r.l. sede di Pavia GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/10	Sala A.	2023	Magnitude of the residue of Boscalid (188425-85-6) in oilseed rape (Raw Agricultural Commodity – RAC) grown in open field conditions after two application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe report no. LBN-0119-2023 LabAnalysis s.r.l. GLP: Yes Published: No	N	Pestila* ProAgri**

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
	Sala A.	2024	Final Report Amendment 1 Magnitude of the residue of Boscalid (188425-85-6) in oilseed rape (Raw Agricultural Commodity – RAC) grown in open field conditions after two application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe report no. LBN-0119-2023 LabAnalysis Life Science s.r.l. sede di Pavia GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/11 (filled as KCP 10.4.1.1/01)	Wesołowska K.	2024	Earthworm Reproduction Test (<i>Eisenia andrei</i>) Report no.: ETOX-2023-26 Source: EcoTox Alliance Sp. z o. o., Poland GLP: Yes Published: No	N	Pestila* ProAgri**
KCP 5.1.2/12 and KCP 5.2/01	Schlewitz P.	2024	Validation of the Analytical Method for the Analysis of Boscalid in Honey Report no.: R C3128 ANADIAG SAS, France GLP: Yes Published: No	N	Pestila* ProAgri**

*Pestila Spółka z ograniczoną odpowiedzialnością (short name Pestila Sp. z o. o.)

** ProAgri International Spółka z ograniczoną odpowiedzialnością or ProAgri Spółka z ograniczoną odpowiedzialnością (short name: ProAgri International Sp. z o. o. or ProAgri Sp. z o. o.)

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	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.	N	BASF

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
			1999/11461 GLP, unpublished MET2001-260		
KCP 5.2	Funk, H. und 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 MET2001-266	N	BASF
KCP 5.2	Funk, H. und Mackenroth, Ch.	2001	Determination of the stability of 205259 (BAS 480 F), 242009 (BAS 49 F), 285028 (BAS 505 F) and 300355 (BAS 510 F) in different solvents; study code 41841. 2000/1014856 GLP, unpublished MET2001-258	N	BASF
KCP 5.2	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 MET2001-267	N	BASF
KCP 5.2	Bross, M.	2001	Investigations on the extractability of 14CBAS 510 F residues from plant matrices; Study code 73479. 2001/1001739 GLP, unpublished MET2001-268	N	BASF
KCP 5.2	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	BASF

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
			MET2001-261		
KCP 5.2	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 MET2001-269	N	BASF
KCP 5.2	Grosshans, F.	2001	The stability of BAS 510F and the metabolites M510F01, M510F49, M510F51 and M510F53 in Acetonitrile; study code 42393. 2000/1017225 GLP, unpublished MET2001-259	N	BASF
KCP 5.2	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, unpublished MET2001-262	N	BASF
KCP 5.2	Keller, W.	1998a	Validation of analytical method no. 408/1, GCMS determination of BAS 510 F active ingredient residues in soil and sediment after methanol extraction; study code 48541. 1998/11314 GLP, unpublished MET2001-263	N	BASF
KCP 5.2	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	BASF

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
			MET2001-264		
KCP 5.2	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 MET2001-265	N	BASF
KCP 5.2	Zangmeister, W.	2000	Validation of analytical method 460, determination of BAS 510 F (Reg.no. 300355) in air by GC-MS; study code 41886. 2000/1014992 GLP, unpublished MET2001-271	N	BASF

The following tables are to be completed by MS

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 1 Detailed evaluation of submitted analytical methods

A 1.1 Analytical methods for the boscalid

A 1.1.1 Methods used for the generation of pre-authorization data (KCP 5.1)

A 1.1.1.1 Description of analytical methods for the determination of active substance and/or variant in the plant protection product

Please refer to the points 5.2.1.1 and 5.2.1.2.

A 1.1.1.2 Description of analytical methods used in ecotoxicological studies

A 1.1.1.2.1 UHPLC-MS/MS (in Elendt M7 medium)

A 1.1.1.2.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/01 (filled as KCP 10.2.1.2/01)

Report *Daphnia* sp., Acute Immobilisation Test, Kolek L., 2024, report no. ETOX-2023-20

Amendment No. 1 *Daphnia* sp., Acute Immobilisation Test Kolek L., 2024, report no. ETOX-2023-20

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The concentration of boscalid in Elendt M7 medium was determined using a ultrahigh performance liquid chromatographic method with tandem mass spectrometry detection (UHPLC-MS/MS). The analytical method involves extracting the sample with water, acetonitrile and a mixture of salts and analysing using UHPLC-MS/MS.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the stand-

and stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the concentration of boscalid in the sample is greater than the concentration of the highest standard solution used to prepare the calibration curve, this sample should be diluted with water so that the estimated concentration is within the range of the calibration curve after this dilution.

4 mL of sample and 4 mL of acetonitrile were mixed. Then 2.6 g \pm 0.2 g of the salt mixture (8:2:2:1, w:w:w:w, magnesium sulfate anhydrous : sodium chloride : sodium citrate dihydrate : sodium hydrogen citrate sesquihydrate) was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 rcf). The upper phase was collected. The collected samples are then analysed using UHPLC-MS/MS.

Chromatographic conditions

UHPLC-MS/MS Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Guard Column Zorbax SB-C18 2.1 \times 5 mm, 1.8 μ m, Column Zorbax SB-C18 RRHT 2.1 \times 50 mm, 1.8 μ m, 600 bar, 6460 Triple Quad Mass Spectrometer (Ion Source AJS ESI)

Injection 2 μ L

Elution Isocratic

Mobile phases 50%: Water + formic acid (0.05%),
50%: Acetonitrile + formic acid (0.05%)

Flow [mL/min] 0.4

Stop time 2.5 min

Column temperature 40 $^{\circ}$ C

MS/MS	Gas Temperature [$^{\circ}$ C]	300
	Gas Flow [L/min]	10
	Nebulizer [psi]	30
	Sheath Gas Heater [$^{\circ}$ C]	320
	Sheath Gas Flow [L/min]	12
	Capillary [V]	3000
	Nozzle Voltage [V]	1000

Transition	Precursor m/z	Product m/z	Dwell	Frag [V]	CE [V]	Cell Acc [V]	Polarity
Target	343	\rightarrow 307.2	150	138	18	2	Positive
Qualifier	343	\rightarrow 271.2	150	138	30	2	Positive

Ion ratio [%] 31% \pm 30% (relative)

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 90 μ g/L standard solution of boscalid to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: 4 mL of Elendta M7 medium and 4 mL of acetonitrile

were mixed. Then 2.5996 g of the salt mixture was added, vigorously shaken by hand and centrifuged (5 min, 5000 rcf). The upper phase was collected. 90 µL of the 1 mg/L boscalid standard solution in acetonitrile was added to 910 µL of collected upper phase.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	18550	18182	789	4.3
	2	17276			
	3	18720			
Spiked matrix blank	1	16554	17377	735	4.2
	2	17610			
	3	17967			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{17377}{18182} - 1 \right) = -4.4\%$$

The matrix effect did not exceed ±20%, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 3, 10, 30, 60, 90 and 120 µg/L.

Standard solutions were prepared by adding a given volume of boscalid stock solution to a volumetric flask and making up to the mark with acetonitrile.

Stock solution 1 g/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

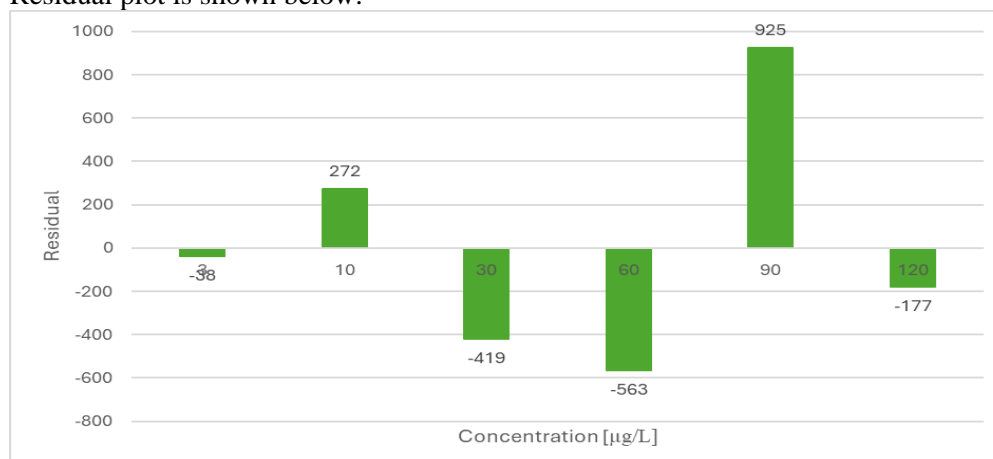
Stock solution 10 mg/L was prepared by taking 100 µL of 1 g/L boscalid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Stock solution 1 mg/L was prepared by taking 1 mL of 10 mg/L boscalid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R ²	Linearity range	Range of analytical method
A = 188.3 C + 308	0.9966	3 – 120 µg/L	≥ 10 µg/L

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 10 µg/L

LOD: 3 µg/L

Precision and accuracy were determined at 2 concentration levels of boscalid in Elendta M7 medium: 10 µg/L (LOQ) and 100 µg/L (10×LOQ).

Five LOQ samples and five 10×LOQ samples were prepared. Sample preparation and analysed 3 times on the same day.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found. Results are shown in the table below.

Determined precision 7.2% (n = 10) meets the acceptance criteria (\leq 20%).

Determined accuracy 90.5% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10×LOQ (100 µg/L)	95.9	3.6	90.5	7.2
	LOQ (10 µg/L)	85.1	3.9		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of two matrix blank samples: 4 mL of Elendta M7 medium and 4 mL of acetonitrile were mixed. Then 2.5984 g or 2.5866 g of the salt mixture was added, vigorously shaken by hand and centrifuged (5 min, 5000 rcf).

Preparation of the matrix spiked sample at LOQ level (10 µg/L): 10 µL of 1000 µg/L boscalid standard solution was added to 0.990 mL of the matrix blank sample.

Preparation of the matrix spiked sample at LOD level (3 µg/L): 3 µL of 1000 µg/L boscalid standard solution was added to 0.997 mL of the matrix blank sample.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

To further confirm the specificity of the analytical method, two ion transitions were recorded:

Target: 343.0 → 307.2

Qualifier: 343.0 → 271.2

Specificity was verified using the ion transition ratio of $31\% \pm 30\%$ (relative). Specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in Elendt M7 medium

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed. Specificity was verified using the ion transition ratio of $31\% \pm 30\%$ (relative). Specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 188.3 C + 308$ Coefficient of determination R^2 : 0.9966 Number of data points: 6
Calibration range	Linearity range: 3 – 120 µg/L Range of analytical method: ≥ 10 µg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 10 µg/L LOD: 3 µg/L

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in Elendt M7 medium.

A 1.1.1.2.2 UHPLC-MS/MS (in AAP medium)

A 1.1.1.2.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/02 (filled as KCP 10.2.1.3/01)
Report	Freshwater Alga and Cyanobacteria, Growth Inhibition Test, Kolek L., 2024, report no. ETOX-2023-21 Amendment No. 1 Freshwater Alga and Cyanobacteria, Growth Inhibition Test, Test, Kolek L., 2024, report no. ETOX-2023-21
Guideline(s):	Yes SANTE/2020/12830, Rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of boscalid in AAP medium was determined using a ultrahigh performance liquid chromatographic method with tandem mass spectrometry detection (UHPLC-MS/MS). The analytical method involves centrifuge the sample and analysing using UHPLC-MS/MS.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the concentration of boscalid in the sample is greater than the concentration of the highest standard solution used to prepare the calibration curve, this sample should be diluted with AAP medium so that the estimated concentration is within the range of the calibration curve after this dilution.

Sample was centrifuged (10 min, 3000 rcf). The upper phase was collected and analysed using UHPLC-MS/MS.

Chromatographic conditions

UHPLC-MS/MS Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Guard Column Zorbax SB-C18 2.1×5 mm, 1.8 µm, Column Zorbax SB-C18 RRHT 2.1×50 mm, 1.8 µm, 600 bar, 6460 Triple Quad Mass Spectrometer (Ion Source AJS ESI)

Injection 10 µL

Elution Isocratic

Mobile phases 50%: Water + formic acid (0.05%),
50%: Acetonitrile + formic acid (0.05%)

Flow [mL/min] 0.4

Stop time 2.5 min

Column temperature 40 °C

MS/MS Gas Temperature [°C] 300
Gas Flow [L/min] 10
Nebulizer [psi] 30
Sheath Gas Heater [°C] 320
Sheath Gas Flow [L/min] 12
Capillary [V] 3000
Nozzle Voltage [V] 1000

Transition	Precursor m/z	Product m/z	Dwell	Frag [V]	CE [V]	Cell Acc [V]	Polarity
Target	343	→ 307.2	150	138	18	2	Positive
Qualifier	343	→ 271.2	150	138	30	2	Positive

Ion ratio [%] 35.2% ± 30% (relative)

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 µg/L standard solution of boscalid in water and AAP medium.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard (water)	1	9697	9477	193	2.0
	2	9397			

	3	9337			
Spiked matrix blank (AAP medium)	1	10773	10862	144	1.3
	2	10785			
	3	11028			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{10862}{9477} - 1 \right) = 14.6\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 0.3, 1, 3, 10, 20, and 30 $\mu\text{g/L}$.

Standard solutions were prepared by adding a given volume of boscalid stock solution to a volumetric flask and making up to the mark with AAP medium.

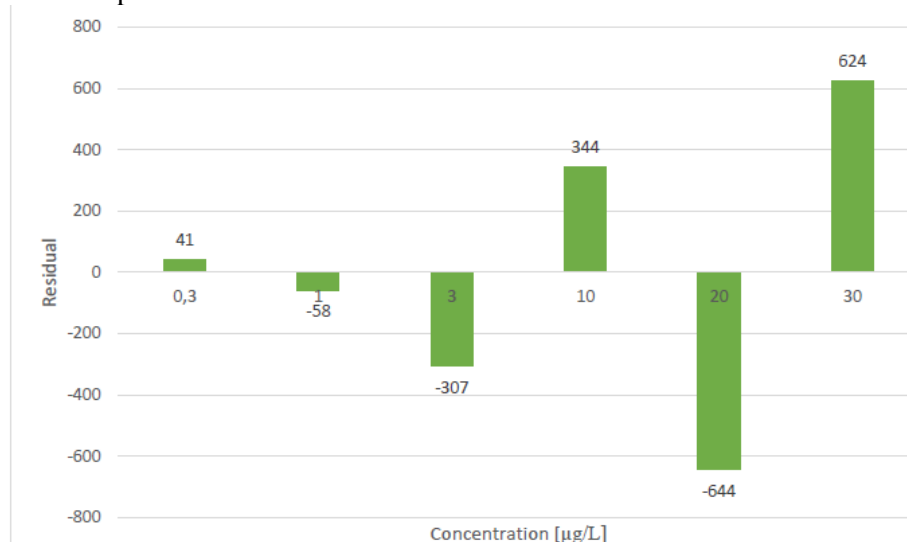
Stock solution 1 g/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Stock solution 1 mg/L was prepared by taking 0.01 mL of 1 g/L boscalid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range	Range of analytical method
$A = 1136 C - 85.4$	0.9988	0.3 – 30 $\mu\text{g/L}$	$\geq 1 \mu\text{g/L}$

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 1 µg/L

LOD: 0.3 µg/L

Precision and accuracy were determined at 2 concentration levels of boscalid in AAP medium: 1 µg/L (LOQ) and 10 µg/L (10×LOQ).

Five LOQ samples and five 10×LOQ samples were prepared. Sample preparation and analysed 3 times on the same day.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). One outlier (10×LOQ2) was found and omitted from the calculations.

Determined precision 1.6% (n = 9) meets the acceptance criteria (\leq 20%).

Determined accuracy 113.6% (n = 9) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
Test Item	10×LOQ (10 µg/L)	114.5 n = 4	0.3 n = 4	113.6 n = 9	1.6 n = 9
	LOQ (1 µg/L)	112.8 n = 5	2.0 n = 5		

Specificity

Representative chromatograms of standard in AAP medium at the lowest calibrated level (LOD) and at LOQ level, matrix blanks (AAP medium) are provided to prove selectivity of the method.

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

To further confirm the specificity of the analytical method, two ion transitions were recorded:

Target: 343.0 → 307.2

Qualifier: 343.0 → 271.2

Specificity was verified using the ion transition ratio of $35.2\% \pm 30\%$ (relative). Specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in AAP medium

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed. Specificity was verified using the ion transition ratio of $35.2\% \pm 30\%$ (relative). Specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 1136 C - 85.4$ Coefficient of determination R^2 : 0.9988 Number of data points: 6
Calibration range	Linearity range: 0.3 – 30 µg/L Range of analytical method: ≥ 1 µg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 1 µg/L LOD: 0.3 µg/L

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in AAP medium.

A 1.1.1.2.3 UHPLC-DAD (in 0.1% Triton X-100 solution)

A 1.1.1.2.3.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/03 (filled as KCP 10.3.1.1.2/02)

Report Bumblebees (*Bombus spp.*), Acute Contact Toxicity Test, Szlauer S., 2023, report no. ETOX-2023-23

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The concentration of boscalid in 0.1% Triton X-100 solution was determined using a ultrahigh performance liquid chromatographic method with DAD detection (UHPLC-DAD). The analytical method involves extracting the sample with acetonitrile and a mixture of salts, dilution with 0.1% formic acid 2 times and analysing using UHPLC-DAD.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the sample concentration is greater than 50 mg/L, this sample shall be diluted with 0.1% Triton X-100 solution so that the estimated concentration is in the range of 2.5 mg/L - 50 mg/L after this dilution.

4 mL of sample and 4 mL of acetonitrile were mixed. Then 2.6 g \pm 0.2 g of the salt mixture (8:2:2:1, w:w:w:w, magnesium sulfate anhydrous : sodium chloride : sodium citrate dihydrate : sodium hydrogen citrate sesquihydrate) was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 ref). 500 μ L of the upper phase was collected and 500 μ L of 0.1% formic acid was added. The samples are then analysed using UHPLC-DAD.

Chromatographic conditions

UHPLC-DAD Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Diode Array Detector G4212A, Guard Column Zorbax SB-C18 2.1 \times 5 mm, 1.8 μ m, Column Zorbax SB-C18 RRHT 2.1 \times 50 mm, 1.8 μ m, 600 bar

Injection 1 μ L

Elution Gradient

Mobile phases A: Water + formic acid (0.05%),
B: Acetonitrile + formic acid (0.05%),

Elution timetable	Time [min]	A:B	Flow [mL/min]
	0.0	50:50	0.4
	2.5	50:50	0.4
	3.0	5:95	0.4
	4.0	5:95	0.4
	4.5	50:50	0.4

Stop time 5.5 min

Column temperature 40 $^{\circ}$ C

DAD Wave length [nm] 254

Band width [nm] 4
Reference wave length [nm] 560
Reference band width [nm] 100
Stop time [min] 2.5

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 mg/L standard solution of Boscalid (the preparation of the solution is described in the paragraph 9.2) to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: 4 mL of 0.1% Triton X-100 solution and 4 mL of acetonitrile were mixed. Then 2.5846 g of the salt mixture was added, vigorously shaken by hand and centrifuged (5 min, 5000 rcf). 490 µL of the upper phase was collected, 10 µL of the 1000 mg/L Boscalid standard solution in acetonitrile was added. 500 µL of 0.1% formic acid was added.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	62.91	62.06	0.73	1.2
	2	61.67			
	3	61.60			
Spiked matrix blank	1	59.49	60.22	0.64	1.1
	2	60.72			
	3	60.45			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{60.22}{62.06} - 1 \right) = -3\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 0.3, 1, 3, 10, 20 and 30 mg/L.

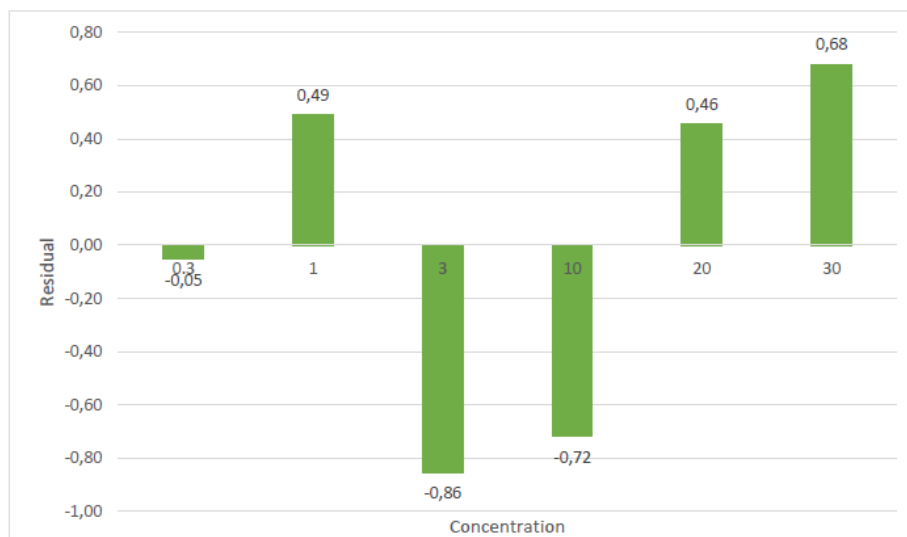
Standard solutions were prepared by diluting the stock solution of boscalid with a 5 mL of 0.1% formic acid and making up to the mark with acetonitrile in a volumetric flask.

Stock solution 1000 mg/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range	Range of analytical method
$A = 6.252 C + 0.261$	0.9997	0.3 – 30 mg/L	≥ 2.5 mg/L

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration)

LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Precision and accuracy were determined at 2 concentration levels of Boscalid (active substance) in 0.1% Triton X-100 solution: 2 mg/L (LOQ) and 20 mg/L of (10 \times LOQ).

Five LOQ samples and five 10 \times LOQ samples were prepared. Sample preparation and analysed 3 times on the same day.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found.

Determined precision 5.0% (n = 10) meets the acceptance criteria (\leq 20%).

Determined accuracy 91.8% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10xLOQ (20 mg/L)	96.0	1.4	91.8	5.0
	LOQ (2 mg/L)	87.6	1.4		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of the matrix blank sample:

4 mL of 0.1% Triton X-100 solution and 4 mL of acetonitrile were mixed. Then 2.5976 g or 2.5861 g of the salt mixture was added (two matrix blank samples were prepared), vigorously shaken by hand and centrifuged (5 min, 5000 rcf). 0.5 mL of the upper phase sample was collected and 0.5 mL of 0.1% formic acid was added.

Preparation of matrix spiked sample at LOD level (0.3 mg/L):

0.497 mL of the upper phase after centrifugation of matrix blank sample was collected, 3 μ L of the 100 mg/L boscalid standard solution in acetonitrile was added. 0.5 mL of 0.1% formic acid was added.

Preparation of matrix spiked sample at LOQ level (1 mg/L):

0.490 mL of the upper phase after centrifugation of matrix blank sample was collected, 10 µL of the 100 mg/L boscalid standard solution in acetonitrile was added. 0.5 mL of 0.1% formic acid was added.

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in 0.1% Triton X-100 solution

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 6.252 C + 0.261$ Coefficient of determination R^2 : 0.9997 Number of data points: 6
Calibration range	Linearity range: 0.3 – 30 mg/L Range of analytical method: ≥ 2.5 mg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration) LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in 0.1% Triton X-100 solution.

A 1.1.1.2.4 UHPLC-DAD (in 50% Sucrose Syrup)

A 1.1.1.2.4.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/04 (filled as KCP 10.3.1.1.1/02)
Report	Bumblebees (<i>Bombus spp.</i>), Acute Oral Toxicity Test, Szlauer S., 2023, report no. ETOX-2023-22
Guideline(s):	Yes SANTE/2020/12830, Rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of boscalid in 50% Sucrose Syrup was determined using a ultrahigh performance liquid chromatographic method with DAD detection (UHPLC-DAD). The analytical method involves extracting the sample with acetonitrile and a mixture of salts, dilution with 0.1% formic acid 2 times and analysing using UHPLC-DAD.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the sample concentration is greater than 50 mg/L, this sample shall be diluted with 50% Sucrose Syrup so that the estimated concentration is in the range of 2.5 mg/L - 50 mg/L after this dilution.

4 mL of sample and 4 mL of acetonitrile were mixed. Then $2.6 \text{ g} \pm 0.2 \text{ g}$ of the salt mixture (8:2:2:1, w:w:w:w, magnesium sulfate anhydrous : sodium chloride : sodium citrate dihydrate : sodium hydrogen citrate sesquihydrate) was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 ref). 500 μL of the upper phase was collected and 500 μL of 0.1% formic acid was added. The samples are then analysed using UHPLC-DAD.

Chromatographic conditions

UHPLC-DAD Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Diode Array Detector G4212A, Guard Column Zorbax SB-C18 2.1 \times 5 mm, 1.8 μm , Column Zorbax SB-C18 RRHT 2.1 \times 50 mm, 1.8 μm , 600 bar

Injection 1 μL

Elution Gradient

Mobile phases A: Water + formic acid (0.05%),
B: Acetonitrile + formic acid (0.05%),

Elution timetable	Time [min]	A:B	Flow [mL/min]
	0.0	50:50	0.4
	2.5	50:50	0.4
	3.0	5:95	0.4
	4.0	5:95	0.4
	4.5	50:50	0.4

Stop time 5.5 min

Column temperature 40 $^{\circ}\text{C}$

DAD	Wave length [nm]	254
	Band width [nm]	4
	Reference wave length [nm]	560
	Reference band width [nm]	100

Stop time [min] 2.5

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 mg/L standard solution of boscalid to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: 4 mL of 50% Sucrose Syrup and 4 mL of acetonitrile were mixed. Then 2.5784 g of the salt mixture was added, vigorously shaken by hand and centrifuged (5 min, 5000 rcf). 490 µL of the upper phase was collected, 10 µL of the 1000 mg/L boscalid standard solution in acetonitrile was added. 500 µL of 0.1% formic acid was added.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	62.91	62.06	0.73	1.2
	2	61.67			
	3	61.60			
Spiked matrix blank	1	62.05	62.06	0.14	0.2
	2	61.93			
	3	62.21			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{62.06}{62.06} - 1 \right) = 0\%$$

The matrix effect did not exceed ±20%, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 0.3, 1, 3, 10, 20 and 30 mg/L.

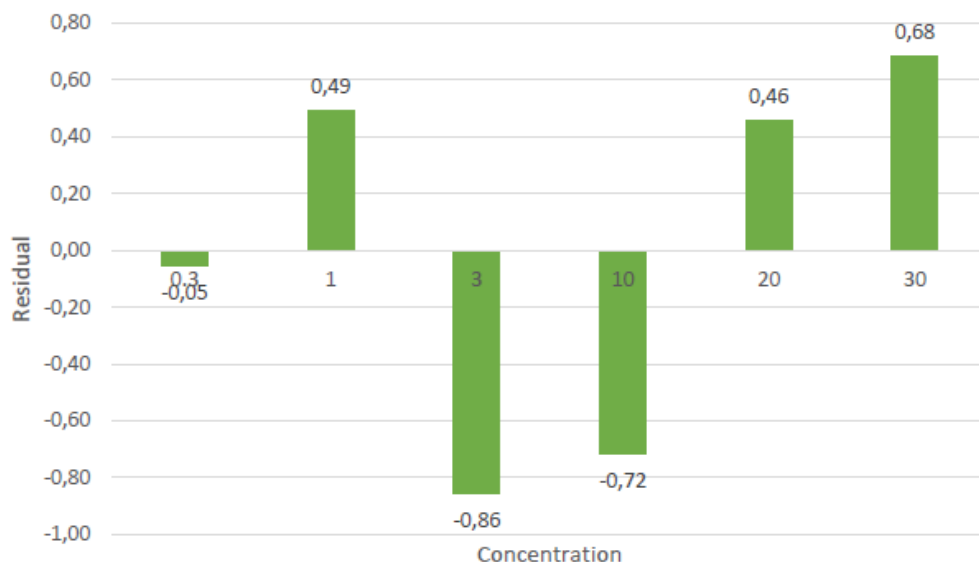
Standard solutions were prepared by diluting the stock solution of boscalid with a 5 mL of 0.1% formic acid and making up to the mark with acetonitrile in a volumetric flask.

Stock solution 1000 mg/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R ²	Linearity range	Range of analytical method
A = 6.252 C + 0.261	0.9997	0.3 – 30 mg/L	≥ 2.5 mg/L

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration)

LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Precision and accuracy were determined at 2 concentration levels of boscalid (active substance) in 50% sucrose syrup: 2 mg/L (LOQ) and 20 mg/L of (10×LOQ).

Five LOQ samples and five 10×LOQ samples were prepared. Sample preparation and analysed 3 times on the same day. Measured concentration after sample preparation is half of the nominal concentration: 10×LOQ 10 mg/L and LOQ 1 mg/L.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found.

Determined precision 4.7% (n = 10) meets the acceptance criteria (\leq 20%).

Determined accuracy 98.4% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10xLOQ (20 mg/L)	102.5	1.6	98.4	4.7
	LOQ (2 mg/L)	94.3	2.2		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of the matrix blank sample:

4 mL of 50% Sucrose Syrup and 4 mL of acetonitrile were mixed. Then 2.6096 g or 2.6011 g of the salt mixture was added (two matrix blank samples were prepared), vigorously shaken by hand and centrifuged (5 min, 5000 rcf). 0.5 mL of the upper phase sample was collected and 0.5 mL of 0.1% formic acid was added.

Preparation of matrix spiked sample at LOD level (0.3 mg/L):

0.497 mL of the upper phase after centrifugation of matrix blank sample was collected, 3 µL of the 100 mg/L Boscalid standard solution in acetonitrile was added. 0.5 mL of 0.1% formic acid was added.

Preparation of matrix spiked sample at LOQ level (1 mg/L):

0.490 mL of the upper phase after centrifugation of matrix blank sample was collected, 10 µL of the 100 mg/L Boscalid standard solution in acetonitrile was added. 0.5 mL of 0.1% formic acid was added.

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in 50% Sucrose Syrup

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 6.252 C + 0.261$ Coefficient of determination R^2 : 0.9997 Number of data points: 6
Calibration range	Linearity range: 0.3 – 30 mg/L Range of analytical method: ≥ 2.5 mg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration) LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in 50% Sucrose Syrup.

A 1.1.1.2.5 UHPLC-DAD (in water)

A 1.1.1.2.5.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/05 (filled as KCP 10.6.2/01)
Report	Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, Wesółowska K., 2024, report no. ETOX-2023-28
Guideline(s):	Yes SANTE/2020/12830, Rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of boscalid in water was determined using a ultrahigh performance liquid chromatographic method with DAD detection (UHPLC-DAD). The analytical method involves dilution with 0.1% formic acid in acetonitrile 2 times and analysing using UHPLC-DAD.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the sample concentration is greater than 50 mg/L, this sample shall be diluted with water so that the estimated concentration is in the range of 2.5 mg/L - 50 mg/L after this dilution.

500 µL of the sample was collected and 500 µL of 0.1% formic acid in acetonitrile was added. The samples were then analysed using UHPLC-DAD.

Chromatographic conditions

UHPLC-DAD Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Diode Array Detector G4212A, Guard Column Zorbax SB-C18 2.1×5 mm, 1.8 µm, Column Zorbax SB-C18 RRHT 2.1×50 mm, 1.8 µm, 600 bar

Injection 1 µL

Elution Gradient

Mobile phases A: Water + formic acid (0.05%),
B: Acetonitrile + formic acid (0.05%),

Elution timetable	Time [min]	A:B	Flow [mL/min]
	0.0	50:50	0.4
	2.5	50:50	0.4
	3.0	5:95	0.4
	4.0	5:95	0.4
	4.5	50:50	0.4

Stop time 5.5 min

Column temperature 40 °C

DAD	Wave length [nm]	254
	Band width [nm]	4
	Reference wave length [nm]	560
	Reference band width [nm]	100
	Stop time [min]	2.5

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 mg/L standard solution of boscalid to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: To 500 µL of the water, 10 µL of the 1000 mg/L boscalid standard solution in acetonitrile was added. 490 µL of 0.1% formic acid in acetonitrile was added.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	62.91	62.06	0.73	1.2
	2	61.67			
	3	61.60			
Spiked matrix blank	1	60.59	60.82	0.22	0.4
	2	61.02			
	3	60.84			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{60.82}{62.06} - 1 \right) = -2.0\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 0.3, 1, 3, 10, 20 and 30 mg/L.

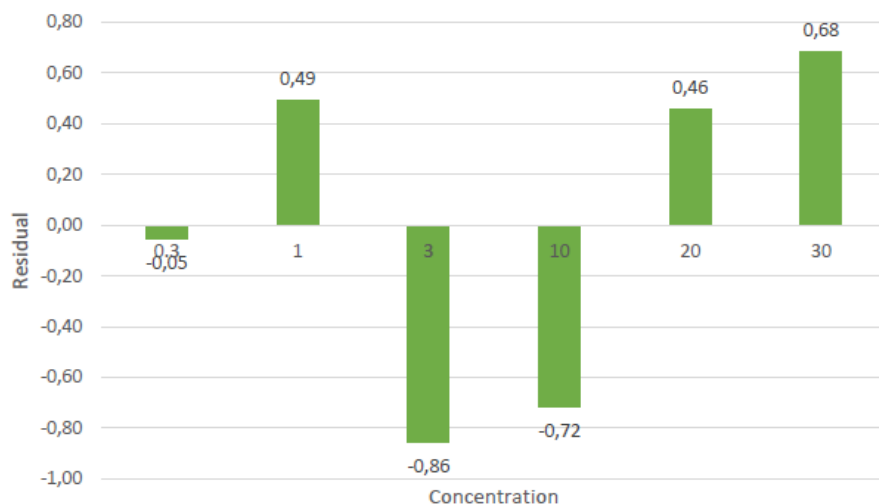
Standard solutions were prepared by diluting the stock solution of boscalid with a 5 mL of 0.1% formic acid and making up to the mark with acetonitrile in a volumetric flask.

Stock solution 1000 mg/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range	Range of analytical method
$A = 6.252 C + 0.261$	0.9997	0.3 – 30 mg/L	≥ 2.5 mg/L

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration)

LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Precision and accuracy were determined at 2 concentration levels of boscalid (active substance) in water: 2 mg/L (LOQ) and 20 mg/L of (10×LOQ).

Five LOQ samples and five 10×LOQ samples were prepared. Sample preparation and analysed 3 times on the same day. Measured concentration after sample preparation is half of the nominal concentration: 10×LOQ 10 mg/L and LOQ 1 mg/L.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found.

Determined precision 2.5% (n = 10) meets the acceptance criteria (\leq 20%).

Determined accuracy 95.7% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10xLOQ (20 mg/L)	97.8	0.5	95.7	2.5
	LOQ (2 mg/L)	93.6	1.4		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of the matrix blank sample:

0.5 mL water was collected and 0.5 mL of 0.1% formic acid in acetonitrile was added.

Preparation of matrix spiked sample at LOD level (0.3 mg/L):

0.5 mL of water was collected, 3 μ L of the 100 mg/L boscalid standard solution in acetonitrile was added. 0.497 mL of 0.1% formic acid in acetonitrile was added.

Preparation of matrix spiked sample at LOQ level (1 mg/L):

0.5 mL of water was collected, 10 μ L of the 100 mg/L boscalid standard solution in acetonitrile was added.

ed. 0.49 mL of 0.1% formic acid in acetonitrile was added.

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in water

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 6.252 C + 0.261$ Coefficient of determination R^2 : 0.9997 Number of data points: 6
Calibration range	Linearity range: 0.3 – 30 mg/L Range of analytical method: ≥ 2.5 mg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration) LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in water.

A 1.1.1.2.6 UHPLC-DAD (in water)

A 1.1.1.2.6.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/06 (filled as KCP 10.6.2/02)
Report	Terrestrial Plant Test: Vegetative Vigour Test, Wesołowska K., 2024, report no. ETOX-2023-29
Guideline(s):	Yes SANTE/2020/12830, Rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of boscalid in water was determined using a ultrahigh performance liquid chromatographic method with DAD detection (UHPLC-DAD). The analytical method involves dilution with 0.1% formic acid in acetonitrile 2 times and analysing using UHPLC-DAD.

The analytical method was validated using standard solutions of boscalid and the test item BSK-FUN 500 SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 5 mL of sample should be taken for chemical analysis. If the sample concentration is greater than 50 mg/L, this sample shall be diluted with water so that the estimated concentration is in the range of 2.5 mg/L - 50 mg/L after this dilution.

500 µL of the sample was collected and 500 µL of 0.1% formic acid in acetonitrile was added. The samples were then analysed using UHPLC-DAD.

Chromatographic conditions

UHPLC-DAD Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Diode Array Detector G4212A, Guard Column Zorbax SB-C18 2.1×5 mm, 1.8 µm, Column Zorbax SB-C18 RRHT 2.1×50 mm, 1.8 µm, 600 bar

Injection 1 µL

Elution Gradient

Mobile phases A: Water + formic acid (0.05%),
B: Acetonitrile + formic acid (0.05%),

Elution timetable	Time [min]	A:B	Flow [mL/min]
	0.0	50:50	0.4
	2.5	50:50	0.4
	3.0	5:95	0.4
	4.0	5:95	0.4
	4.5	50:50	0.4

Stop time 5.5 min

Column temperature 40 °C

DAD	Wave length [nm]	254
	Band width [nm]	4
	Reference wave length [nm]	560
	Reference band width [nm]	100
	Stop time [min]	2.5

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 mg/L standard solution of boscalid to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: To 500 µL of the water, 10 µL of the 1000 mg/L boscalid standard solution in acetonitrile was added. 490 µL of 0.1% formic acid in acetonitrile was added.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	62.91	62.06	0.73	1.2
	2	61.67			
	3	61.60			
Spiked matrix blank	1	60.59	60.82	0.22	0.4
	2	61.02			
	3	60.84			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{60.82}{62.06} - 1 \right) = -2.0\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of boscalid at the concentrations of 0.3, 1, 3, 10, 20 and 30 mg/L.

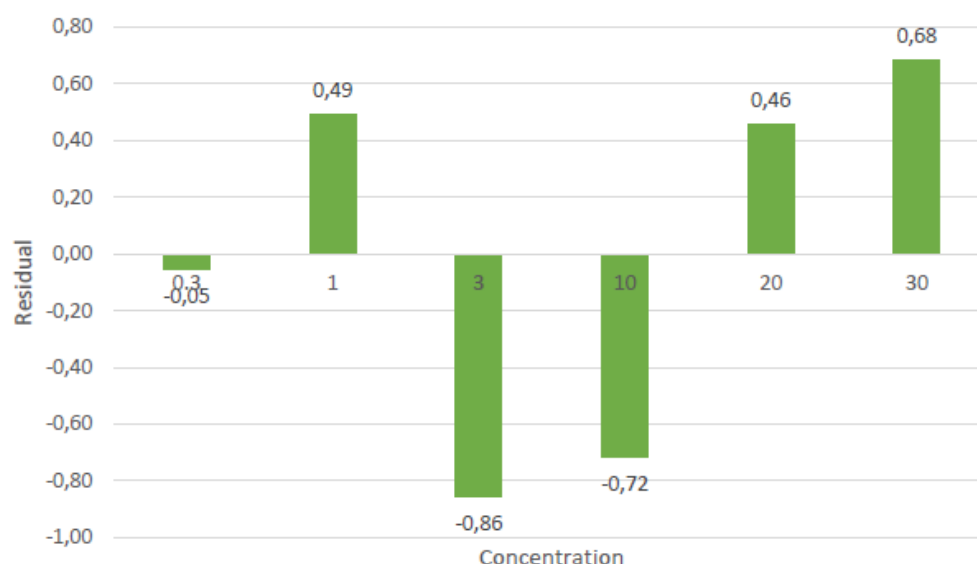
Standard solutions were prepared by diluting the stock solution of boscalid with a 5 mL of 0.1% formic acid and making up to the mark with acetonitrile in a volumetric flask.

Stock solution 1000 mg/L was prepared by weighing 10 mg of the boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range	Range of analytical method
$A = 6.252 C + 0.261$	0.9997	0.3 – 30 mg/L	≥ 2.5 mg/L

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) \leq 20%). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration)

LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Precision and accuracy were determined at 2 concentration levels of boscalid (active substance) in water: 2 mg/L (LOQ) and 20 mg/L of (10 \times LOQ).

Five LOQ samples and five 10 \times LOQ samples were prepared. Sample preparation and analysed 3 times on the same day. Measured concentration after sample preparation is half of the nominal concentration: 10 \times LOQ 10 mg/L and LOQ 1 mg/L.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q (α = 0.95). No outliers were found.

Determined precision 2.5% (n = 10) meets the acceptance criteria (\leq 20%).

Determined accuracy 95.7% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10xLOQ (20 mg/L)	97.8	0.5	95.7	2.5
	LOQ (2 mg/L)	93.6	1.4		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of the matrix blank sample:

0.5 mL water was collected and 0.5 mL of 0.1% formic acid in acetonitrile was added.

Preparation of matrix spiked sample at LOD level (0.3 mg/L):

0.5 mL of water was collected, 3 μ L of the 100 mg/L boscalid standard solution in acetonitrile was added. 0.497 mL of 0.1% formic acid in acetonitrile was added.

Preparation of matrix spiked sample at LOQ level (1 mg/L):

0.5 mL of water was collected, 10 μ L of the 100 mg/L boscalid standard solution in acetonitrile was added. 0.49 mL of 0.1% formic acid in acetonitrile was added.

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in water

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 6.252 C + 0.261$ Coefficient of determination R^2 : 0.9997 Number of data points: 6
Calibration range	Linearity range: 0.3 – 30 mg/L Range of analytical method: \geq 2.5 mg/L

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 2 mg/L (nominal concentration) – 1 mg/L (measured concentration) LOD: 0.6 mg/L (nominal concentration) – 0.3 mg/L (measured concentration)

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in water.

A 1.1.1.2.7 UHPLC-TOF-MS/MS (in sucrose solution)

A 1.1.1.2.7.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/07 (filled as KCP 10.3.1.2/01)
Report	Effects of BSK-FUN 500 SC on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Chronic Oral Toxicity Test, Mautino G., 2024, report no. 1142.1F.SAG23
Guideline(s):	SANTE/2020/12830 rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The content of boscalid active ingredient was determined in the lowest concentration and in the highest concentration of the feeding solutions prepared in the biological phase of the study.

The analytical method for boscalid in sucrose solutions was fully validated in this study according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).

Boscalid was extracted from the sucrose solution matrix with acetonitrile, after adding an opportune amount of water. After salts addition, the acetonitrile phase was separated from the aqueous phase.

The final analysis was performed in positive ionisation mode by Ultra High-Performance Liquid Chromatography, tandem High Resolution Mass Spectrometry (UHPLC-TOF-MS/MS).

Two ions were acquired from the parent ion 343.04: the fragment ion 307.06 (target) for quantification purpose and the fragment ion 139.99 (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: UHPLC Series 1290 Infinity Agilent
- MS/MS detector System: Triple TOF 4600 AB SCIEX
- Analytical Column: Zorbax Eclipse plus C18, 1.8 µm, 50 x 2.1 mm, AGILENT
- Mobile phases:

Solvent A: Water (milliQ), 0.1% formic acid, 5mM ammonium formate

- Solvent B: MeOH (LC-MS), 0.1% formic acid
- Pump gradient:
 - 0 min: A 90 % - B 10 %
 - 0.5 min: A 90 % - B 10 %
 - 2 min: A 10 % - B 90 %
 - 4 min: A 10 % - B 90 %
 - 4.5 min: A 10 % - B 90 %
 - 5 min: A 90 % - B 10 %
 - Flow rate: 0.5 mL/min
 - Column Temperature: 40°C
 - Injection volume: 2 µL
 - Retention time: Boscalid: 2.3 minutes
 - TOF-MS/MS: Experiment 1
 - Duration: 5 min
 - Delay: 0 sec
 - Scan type: positive product ion
 - Temperature (TEM): 500°C
 - Curtain gas (CUR): 10 psi
 - Ion Spray Voltage (IS): 5500 V
 - Gas 1: 30 psi
 - Gas 2: 30 psi
 - TOF mass range: 100-2000 Da
 - Accumulation time: 0.1 sec
 - DP: 130
 - CE 25
 - Ions:
 - Boscalid-1 (primary ion): m/z (307.0000-307.1000)
 - Boscalid-2 (confirmatory ion): m/z (139.9000-140.0000)

Stock and working reference item solutions

20.6 mg of the reference item were weighed into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at 1019.7 µg/mL; stored in freezer at a nominal temperature of -18 °C).

A second stock solution of boscalid reference item was prepared by weighing 21.0 mg into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at 1039.5 µg/mL; stored in freezer at a nominal temperature of -18 °C).

By dilution of the reference item stock solutions described above in acetonitrile reference item working solutions were prepared, as reported in the table below.

Boscalid reference item parent solution concentration (µg/mL)	Taken Volume (mL)	Final Volume (mL)	Boscalid reference item working solution concentration (µg/mL)	Solution type
1019.7	0.1	10	10.197	Intermediate
10.197	1	10	1.0197	Intermediate
1039.5	0.1	10	10.395	Intermediate
10.395	0.025	1	0.25988	Stability check

The boscalid standard solutions were freshly prepared on the same analysis day.

Test item solutions and fortification procedure for sucrose solution matrix

508.5 mg of the test item were weighed into a 25 mL volumetric flask and diluted to volume with pure water. This test item solution (containing 8721.6 µg/mL of boscalid active ingredient) was used as recovery solution at the second fortification level. By dilution of this test item solution (0.3 mL in 10 mL pure water) the fortification solution for the 261.65 µg/mL of boscalid LOQ level was obtained.

Fortification procedure

The fortification procedure is described in the following table.

Matrix	Matrix Weight (g)	Fortification level (mg/kg)	Spiking solution concentration (µg/mL)	Spiking solution volume (mL)
Sucrose solutions	2.5	52.330 (LOQ) 872.16 (II level)	261.65 8721.6	0.5 0.25

Analytical procedure for sample extracts preparation: matrix sucrose solution

The sample was let to warm up to room temperature, vigorously shaken by hand for a minute and immediately weighed.

The homogenised sample (2.5 ± 0.05 g) was weighted (with 0.05 mg accuracy) into a 50 mL centrifuge tube; recovery samples were fortified at this point.

Then 7.5 mL of demineralised water were added and the sample was manually shaken for a few seconds to homogenise.

Then 10 mL of acetonitrile were added. In recovery trials, the 10 mL volume comprises the volume of the fortification solution added.

The tube was shaken vigorously by hand for 1 minute. After this step, the content of a sachet of QuEChERS was added to the sample.

The tube is shaken vigorously by hand for 1 minute and then centrifuged at 4000 rpm for five minutes.

The extract was filtered with PTFE filter, porosity 0.20 µm, and finally analysed by HPLC-TOF-MS/MS. Quantification was performed using solvent calibration standards in the concentration range 25 -250 ng/mL.

Sample extracts with analyte concentration exceeding 200 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20 % of the calibration range.

Validation

Linearity

The linearity range for boscalid was found between 25 - 250 ng/mL corresponding to 0.10197 – 1.0197 mg/kg of boscalid in sucrose solution samples. The correlation coefficients of the weighed linear (1/x) multipoint external standard solvent calibration curves were found > 0.992 in all the analytical sequences performed.

The linearity range comprised the concentration range from < 20 % of the LOQ to 20 % above the highest measured concentration.

The suitability of the calibration lines for the matrix sucrose solution was assessed using the residuals d_i that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - y_{yi}$$

where:

y_i is the measured value i ;

y_{yi} is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if d_i were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.

The linearity range comprised the concentration range of the samples $\pm 20\%$.

Blank and selectivity

At least two independent analyses of the blank sample were performed: no significant interference exceeding 20 % of the limit of quantification were found at the retention time of boscalid for both the monitored ions.

Therefore, boscalid can be regarded as not detectable in untreated sucrose solution sample used in fortification trials (< 20 % of LOQ).

The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.

Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of boscalid in sucrose solution, without significant interferences above 20 % of LOQ.

Specificity

Boscalid was analysed by UHPLC-TOF-MS/MS highly specific detection system; two ions were simultaneously acquired: the ion 307.06 (target) for quantification purpose and the ion 139.99 (qualifier) for confirmation purpose.

The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-2000 m/z.

Recoveries

The analytical method for sucrose solution matrix was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.

The recoveries for matrix sucrose solution were performed by fortifying the untreated blank at two levels. For matrix sucrose solution, the recoveries were performed by fortifying the untreated blank at two levels. The LOQ level was set at boscalid concentration of 52.330 mg/kg, (lower than the nominal boscalid content in the lowest concentration dose), while the second level was at 872.16 mg/kg (higher than the maximum expected concentration in the samples) in order to cover with the method validation all the range of boscalid concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.

The background content in the sucrose solution control sample used in fortification experiments was not detectable.

In these recovery samples the boscalid content was determined as reported in table below.

Recovery tests results for boscalid in sucrose solution (target ion 307.06)

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
52.330 (a) (LOQ)	74.1 89.9 85.9 85.3 84.4	83.9	7.0	89.7 \pm 10.4
872.16 (b) (II level)	87.9 90.8 89.0 105.6 104.5	95.6	9.1	

(a) 122.04 mg/kg as test item

(b) 2034.0 mg/kg as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy

The accuracy of the analysis method for boscalid in sucrose solution matrix, defined as overall mean recovery \pm relative standard deviation, is 89.7 \pm 10.4.

Repeatability

The repeatability of the methods, defined as the % RSD (Relative Standard Deviation) at each fortification level, and the overall RSD is reported in the table below.

Repeatability for boscalid in sucrose solution matrix (target ion 307.06)

Fortification level (mg/kg)	RSD (%) (n=5)	Overall RSD % (n=10)
52.330 (a) (LOQ)	7.0	10.4
872.16 (b) (II level)	9.1	

(a) 122.04 mg/kg as test item

(b) 2034.0 mg/kg as test item

For each fortification level, the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.

The LOQ for boscalid in sucrose solution matrix was assessed in this study at 52.330 mg/kg (122.04 mg/kg referred as test item).

Limit of detection (LOD)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.

For the analysis of boscalid in sucrose solution matrix the LOD is 0.10197 mg/kg. This value was calculated from the boscalid concentration corresponding to the lowest calibration point.

Matrix effects

To check possible signal enhancement or suppression effects in the LC-MS/MS analysis, the sucrose solution control samples extract was fortified to achieve the nominal concentration of boscalid at the LOQ level; the analyte response in this fortified extract was compared with that of boscalid in solvent at the same concentration. The results are summarized in the following table.

Matrix	Ions	Area (counts) solvent stand- ard	Area (counts) matrix matched standard	Matrix re- sponse over solvent re- sponse %	Matrix effects %
Sucrose solutions	307.06 (Target)	27051.4	24331.3	90	-10
	139.99 (Qualifier)	1832.1	1629.3	89	-11

No significant matrix effect (i.e. exceeding ± 20 %) was found for boscalid in sucrose solution matrix, then solvent calibration standards were used for quantification of samples.

Confirmation

The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional qualifier ion for confirmation.

The recovery data and the precision data for the additional transition in sucrose solution matrix are reported respectively in table below.

Data for boscalid in matrix sucrose solution (qualifer ion 139.99)

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
52.330 (a) (LOQ)	96.5	87.0	7.0	90.5 \pm 8.9
	88.1			
	83.5			
	86.4			
	80.5			

872.16 (b) (II level)	87.1 91.9 85.0 100.3 105.8	94.0	9.4	
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(a) 122.04 mg/kg as test item

(b) 2034.0 mg/kg as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability of final extracts and reference item solutions

The final extracts were analysed within 24 hours form extraction; moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.

The boscalid reference item stock solution was proven to be stable for 5 days after preparation in frozen conditions: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ -18 °C in the dark) did not differ by more than 10%.

Conclusion

Boscalid (target ion 307.06)				
Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
52.330 (a) (LOQ)	74.1 89.9 85.9 85.3 84.4	83.9	7.0	89.7 ± 10.4
872.16 (b) (II level)	87.9 90.8 89.0 105.6 104.5	95.6	9.1	
Limits of the method Limit of quantification: 52.330 mg/kg Limit of detection: 0.10197 mg/kg Linearity range: from 25 to 250 ng/mL Validation range: 52.33 – 872.16 mg/kg (corresponding to 0.10197 – 1.0197 mg/kg in samples at the minimum dilution) r ≥ 0.992				

(a) 122.04 mg/kg as test item

(b) 2034.0 mg/kg as test item

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of boscalid active ingredient in sucrose solutions in the range 52.330 – 872.16 mg/kg (corresponding to 122.04 – 2034.0 mg/kg referred as test item).

As the analysis of the samples was performed during the recovery tests, the reliability of the found values was demonstrated.

A 1.1.1.2.8 UHPLC-TOF-MS/MS (in water)

A 1.1.1.2.8.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/08 (filled as KCP 10.3.1.4/01)
Report	Effects of BSK-FUN 500 SC on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure, Mautino G., 2024, report no. 1143.1F.SAG23
Guideline(s):	SANTE/2020/12830 rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The content of boscalid active ingredient was determined in the lowest concentration and in the highest concentration of the water stock solutions prepared in the biological phase of the study.

The analytical method for boscalid in water was fully validated in this study according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).

Boscalid was determined in water stock solutions after sample dilution with acetonitrile and the final analysis was performed in positive ionisation mode by Ultra High-Performance Liquid Chromatography, tandem High Resolution Mass Spectrometry (UHPLC-TOF-MS/MS).

Two ions were acquired from the parent ion 343.04: the fragment ion 307.06 (target) for quantification purpose and the fragment ion 139.99 (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: UHPLC Series 1290 Infinity Agilent
- MS/MS detector System: Triple TOF 4600 AB SCIEX
- Analytical Column: Zorbax Eclipse plus C18, 1.8 µm, 50 x 2.1 mm, AGILENT
- Mobile phases:
 - Solvent A: Water (milliQ), 0.1% formic acid, 2mM ammonium formate
 - Solvent B: MeOH (LC-MS), 0.1% formic acid
- Pump gradient:
 - 0 min: A 90 % - B 10 %
 - 0.5 min: A 90 % - B 10 %
 - 2 min: A 10 % - B 90 %
 - 4 min: A 10 % - B 90 %
 - 4.5 min: A 90 % - B 10 %
 - 5 min: A 90 % - B 10 %
- Flow rate: 0.5 mL/min
- Column Temperature: 40°C
- Injection volume: 2 µL
- Retention time: Boscalid: 2.3 minutes
- TOF-MS/MS: Experiment 1
 - Duration: 5 min
 - Delay: 0 sec
 - Scan type: positive product ion
 - Temperature (TEM): 500°C
 - Curtain gas (CUR): 10 psi
 - Ion Spray Voltage (IS): 5500 V
 - Gas 1: 30 psi
 - Gas 2: 30 psi

TOF mass range: 100-2000 Da
Accumulation time: 0.1 sec
DP: 130
CE 25

– Ions:

Boscalid-1 (primary ion): m/z (307.0000-307.1000)
Boscalid-2 (confirmatory ion): m/z (139.9000-140.0000)

Stock and working reference item solutions

20.6 mg of the reference item were weighed into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at 1019.7 µg/mL; stored in freezer at a nominal temperature of -18 °C).

By dilution of the reference item stock solution described above in acetonitrile reference item working solutions were prepared, as reported in the table below.

Boscalid reference item parent solution concentration (µg/mL)	Taken Volume (mL)	Final Volume (mL)	Boscalid reference item working solution concentration (µg/mL)	Solution type
1019.7	0.1	10	10.197	Intermediate
10.197	1	10	1.0197	Intermediate

The Boscalid standard solutions were freshly prepared on the same analysis day.

Stock and working test item solutions (fortification procedure)

503.4 mg of the test item were weighed into a 25 mL volumetric flask and diluted to volume with pure water. This test item solution (containing 8721.6 µg/mL of boscalid active ingredient) was used as recovery solution at the second fortification level. By dilution of this test item solution (0.6 mL in 10 mL pure water) the solution for the 0.5233 g/L boscalid LOQ level was obtained.

Reference item matrix matched working solution

A reference item matrix matched working solution at 50 ng/mL was prepared to check matrix effect by using as solvent, instead of acetonitrile, the untreated water matrix (undiluted).

Analytical procedure for sample extracts preparation

The sample was let to warm up to room temperature, vigorously shaken by hand for a minute and immediately 1 mL is taken and brought to 10 mL with acetonitrile.

Samples with analyte concentration exceeding 200 ng/mL were opportunely diluted with ACN to fit within the calibrated range.

Recovery samples at LOQ were prepared by taking 1 mL of the solution at LOQ level and reaching 10 mL with acetonitrile. Recovery samples at second level were prepared by taking 1 mL of the solution at second level and reaching 10 mL with acetonitrile.

Recovery samples were opportunely diluted with ACN in order to fit within the calibrated range.

The extract was finally analysed by HPLC-TOF-MS/MS.

Quantification was performed using solvent calibration standards in the concentration range 25 -250 ng/mL.

Validation

Linearity

The linearity range for boscalid was found between 25 - 250 ng/mL corresponding to 0.00025493 to 0.0025493 g/L of boscalid in water sample. The correlation coefficient of the weighed linear (1/x) multipoint external standard solvent calibration curve was found ≥ 0.995 in the analytical sequence performed.

The linearity range comprised the concentration range from < 20 % of the LOQ (taking into account the operative dilution of LOQ level) to at least 20 % above the highest measured concentration.

The suitability of the calibration lines for the water matrix was assessed using the residuals d_i that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - \hat{y}_i$$

where:

y_i is the measured value i ;

\hat{y}_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if d_i were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.

The linearity range comprised the concentration range of the samples ± 20 %.

Blank and selectivity

Two independent analyses of the blank sample were performed: no significant interference exceeding 20 % of the limit of quantification were found at the retention time of boscalid for both the monitored ions. Therefore, boscalid can be regarded as not detectable in untreated water sample used in fortification trials (< 20 % of LOQ).

The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.

Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of boscalid in water solution matrix, without significant interferences above 20 % of LOQ (taking into account the operative dilution of LOQ level).

Specificity

Boscalid was analysed by UHPLC-TOF-MS/MS highly specific detection system; two ions were simultaneously acquired: the ion 307.06 (target) for quantification purpose and the ion 139.99 (qualifier) for confirmation purpose.

The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-2000 m/z.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.

The recoveries were performed by fortifying the untreated blank at two levels.

The LOQ level was set at boscalid concentration of 0.5233 g/L (1.2204 g/L as test item), lower than the minimum found boscalid content in samples, while the second level was at 8.7216 g/L (20.340 g/L as test item), higher than the maximum expected concentration in the samples, in order to cover with the method validation all the range of boscalid concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.

The background content in the control sample used for dilution of test item in fortification experiments was not detectable.

In these recovery samples the boscalid content was determined as reported in table below.

Recovery tests results for boscalid in water matrix (target ion 307.06)

Fortification level (g/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.5233 (a) (LOQ)	95.6 111.6 111.3 111.9 116.0	109.3	7.2	107.5 \pm 5.5
8.7216 (b) (II level)	101.7 106.1 103.9 106.8	105.7	3.0	

	110.1			
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(a) 1.2204 g/L as test item

(b) 20.340 g/L as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy

The accuracy of the analysis method for boscalid in water, defined as mean recovery \pm relative standard deviation, is 107.5 ± 5.5 .

Repeatability

The repeatability, defined as the % RSD (Relative Standard Deviation) at each fortification level, and the overall RSD of water matrix are reported in table below.

Repeatability for boscalid in water matrix (target ion 307.06)

Fortification level (g/L)	RSD (%) (n=5)	Overall RSD % (n=10)
0.5233 (a) (LOQ)	7.2	5.5
8.7216 (b) (II level)	3.0	

(a) 1.2204 g/L as test item

(b) 20.340 g/L as test item

For each fortification level, the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.

The LOQ for boscalid in water was assessed in this study at 0.5233 g/L.

Limit of detection (LOD)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.

For the analysis of boscalid in water the LOD is 0.00025493 g/L. This value was calculated from the boscalid concentration corresponding to the lowest calibration point (undiluted sample).

Matrix effects

To check possible signal enhancement or suppression effects in the LC-MS/MS analysis, the control sample was fortified to achieve the nominal concentration of boscalid at 50 ng/mL; the analyte response in this fortified extract was compared with that of boscalid in solvent at the same concentration. The results are summarized in the following table.

Matrix	Ions	Area (counts) solvent standard	Area (counts) matrix matched standard	Matrix response over solvent response %	Matrix effects %
Sucrose solutions	307.06 (Target)	29710.5	32134.7	108	+8
	139.99 (Qualifier)	2062.9	2229.3	108	+8

No significant matrix effect (i.e., exceeding ± 20 %) was found. For the quantification of water samples, solvent calibration standards were used.

Confirmation

The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.

The recovery data and the precision data for the additional transition are reported in table below.

Data for boscalid (qualifier ion 139.99)

Fortification level (g/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.5233 (a) (LOQ)	95.2 109.5 113.5 116.0 118.0	110.4	8.2	107.5 \pm 6.6
8.7216 (b) (II level)	100.4 104.3 104.2 104.4 109.4	104.5	3.1	

(a) 1.2204 g/L as test item

(b) 20.340 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability of final extracts and reference item solutions

The final extracts were analysed within 24 hours form extraction; moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.

The stability of boscalid reference item stock solution was verified for 5 days after preparation at $\leq -18^{\circ}\text{C}$ in the dark in the Renolab study 23450-01R (1142.1F.SAG23).

Conclusion

Boscalid (target ion 307.06)				
Fortification level (g/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.5233 (a) (LOQ)	95.6 111.6 111.3 111.9 116.0	109.3	7.2	107.5 \pm 5.5
8.7216 (b) (II level)	101.7 106.1 103.9 106.8 110.1	105.7	3.0	
Limits of the method Limit of quantification: 0.5233 g/L Limit of detection: 0.0002549 g/L Linearity range: from 25 to 250 ng/mL Validation range: 0.5233 – 8.722 g/L (corresponding to 0.0002549 - 0.002549 g/L in undiluted samples) $r \geq 0.995$				

(a) 1.2204 g/L as test item

(b) 20.340 g/L as test item

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of boscalid active ingredient in water samples in the range 0.52330 – 8.7216 g/L (corresponding to 1.2204 – 20.340 g/L as test item).

As the analysis of the samples was performed during the recovery tests, the reliability of the found values is demonstrated.

A 1.1.1.2.9 UHPLC-MS/MS (in artificial soil)

A 1.1.1.2.9.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/11 (filled as KCP 10.4.1.1/01)

Report Earthworm Reproduction Test (*Eisenia andrei*); Wesołowska K; 2024; report no.: ETOX-2023-26

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The concentration of Boscalid in artificial soil was determined using a ultrahigh performance liquid chromatographic method with tandem mass spectrometry detection (UHPLC-MS/MS). The analytical method involves extracting the sample with water, acetonitrile and a mixture of salts and analysing using UHPLC-MS/MS.

The analytical method was validated using standard solutions of Boscalid and the Test Item BSK-FUN 500SC. The analytical method was validated according to SANTE/2020/12830, Rev.2.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD) and specificity were assessed in the process of the analytical method validation. Sample will be analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 2 g of sample should be taken for chemical analysis. 4 mL of water and 4 mL of acetonitrile were added to 1.3 g ± 0.3 g of sample and vigorously shaken by hand (approximately 1 min). Then 2.6 g ± 0.2 g of the salt mixture (8:2:2:1, w:w:w:w, magnesium sulfate anhydrous : sodium chloride : sodium citrate dihydrate : sodium hydrogen citrate sesquihydrate) was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 rcf). The upper phase was collected. If the concentration of Boscalid in the upper phase is greater than the concentration of the highest standard solution used to prepare the calibration curve, this sample should be diluted with acetonitrile so that the estimated concentration is within the range of the calibration curve after this dilution. The collected samples are then analysed using UHPLC-MS/MS.

Dry weight of artificial soil for each sample was determined in parallel to the LC-MS/MS analysis.

Chromatographic conditions

UHPLC-MS/MS Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Guard Column Zorbax SB-C18 2.1×5 mm, 1.8 µm, Column Zorbax SB-C18 RRHT 2.1×50 mm, 1.8 µm, 600 bar, 6460 Triple Quad Mass Spectrometer (Ion Source AJS ESI)

Injection 2 µL

Elution Isocratic

Mobile phases 50%: Water + formic acid (0.05%),
50%: Acetonitrile + formic acid (0.05%)

Flow [mL/min] 0.4

Stop time 2.5 min

Column temperature 40 °C

MS/MS

Gas Temperature [°C]	300
Gas Flow [L/min]	10
Nebulizer [psi]	30
Sheath Gas Heater [°C]	320
Sheath Gas Flow [L/min]	12
Capillary [V]	3000
Nozzle Voltage [V]	1000

Transition	Precursor m/z		Product m/z	Dwell	Frag [V]	CE [V]	Cell Acc [V]	Polarity
Target	343	→	307.2	150	138	18	2	Positive
Qualifier	343	→	271.2	150	138	30	2	Positive

Ion ratio [%] 31% ± 30% (relative)

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 90 µg/L standard solution of Boscalid (the preparation of the solution is described in the paragraph 8.2) to spiked matrix blank sample at the same concentration. Results are show in table below.

Preparation of the spiked matrix blank sample: 1.3433 g of artificial soil was weighted. Then 4 mL of water and 4 mL of acetonitrile were added and vigorously shaken by hand (approximately 1 min). Then 2.5870 g of the salt mixture was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 ref). The upper phase was collected. 90 µL of the 1 mg/L Boscalid standard solution in acetonitrile was added to 910 µL of collected upper phase.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	18550	18182	789	4.3
	2	17276			
	3	18720			
Spiked	1	17093	17714	613	3.5

matrix blank	2	18319			
	3	17730			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{17714}{18182} - 1 \right) = -2.6\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of Boscalid at the concentrations of 3, 10, 30, 60, 90 and 120 $\mu\text{g/L}$.

Standard solutions were prepared by adding a given volume of Boscalid stock solution to a volumetric flask and making up to the mark with acetonitrile.

Stock solution 1 g/L was prepared by weighing 10 mg of the Boscalid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

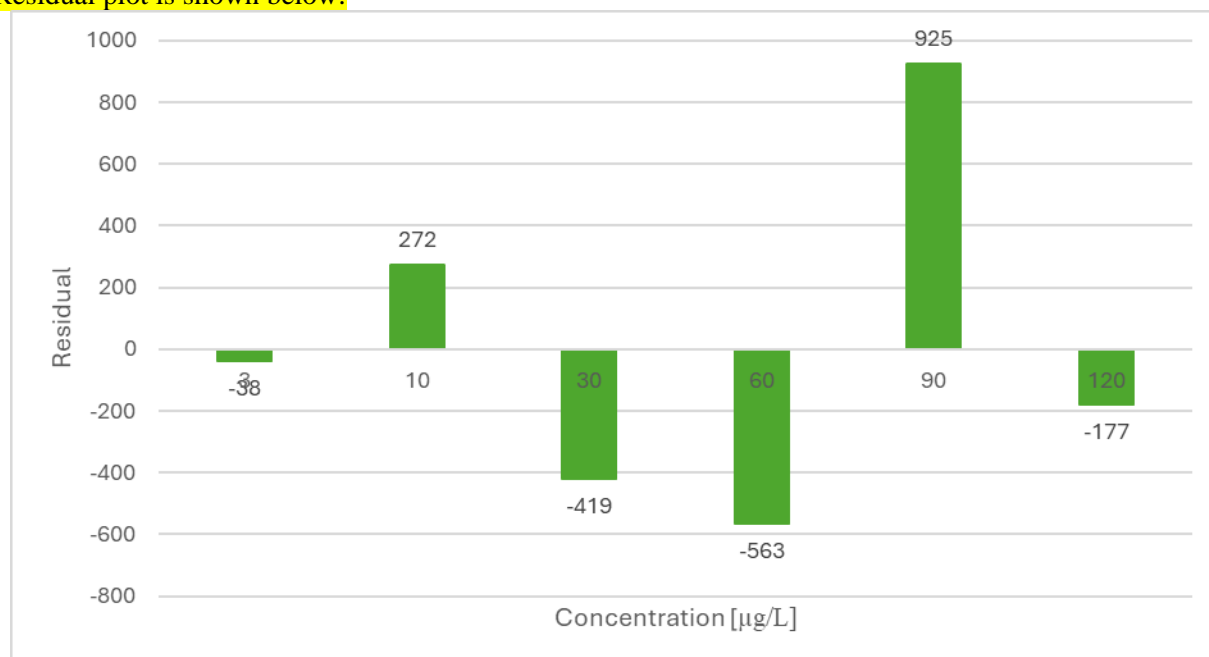
Stock solution 10 mg/L was prepared by taking 100 μL of 1 g/L Boscalid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Stock solution 1 mg/L was prepared by taking 1 mL of 10 mg/L Boscalid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range	Range of analytical method
$A = 188.3 C + 308$	0.9966	3 – 120 $\mu\text{g/L}$	43 $\mu\text{g/kg}$ – 514.5 mg/kg

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) $\leq 20\%$). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 43 $\mu\text{g/kg}$

LOD: 13 $\mu\text{g/kg}$

Precision and accuracy were determined at 2 concentration levels of the Test Item BSK-FUN 500SC in

artificial soil: 0.1 mg/kg (LOQ) and 1200 mg/kg (MAX).

Five LOQ samples and five MAX samples were prepared. Sample preparation and analysed 3 times on the same day. MAX samples were diluted 5000 times.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found.

Determined precision 3.0% (n = 10) meets the acceptance criteria ($\leq 20\%$).

Determined accuracy 99.0% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Accuracy [%]	Precision [%]
Test Item	0.1 mg/kg (LOQ)	100.5	3.3	99.0	3.0
	1200 mg/kg (MAX)	97.5	1.7		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of two matrix blank samples: 1.0110 g or 1.0160 g of dry artificial soil was weighted. Then 4 mL of water and 4 mL of acetonitrile were added and vigorously shaken by hand (approximately 1 min). Then 2.5888 g or 2.6111 g of the salt mixture was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 rcf).

Preparation of the matrix spiked sample at LOQ level (10 µg/L): 10 µL of 1 mg/L Boscalid standard solution was added to 0.990 mL of the matrix blank sample.

Preparation of the matrix spiked sample at LOD level (3 µg/L): 3 µL of 1 mg/L Boscalid standard solution was added to 0.997 mL of the matrix blank sample.

The 3 µg/L concentration corresponds to the lowest concentration on the calibration curve and is equal to 30% of the 10 µg/L concentration.

The signal of the detected substance was overlapping with the matrix signal of the matrix blank samples under the experimental conditions. The peak area on the chromatogram of LOQ level sample is equal to 2444. The peak area on the matrix blank chromatograms is equal to 580 and 564 which is 23.7% and 23.1% of LOQ level, respectively. Matrix blank signal was not higher than 30% of the LOQ, which is acceptable.

To further confirm the specificity of the analytical method, two ion transitions were recorded:

Target: 343.0 → 307.2

Qualifier: 343.0 → 271.2

Specificity was verified using the ion transition ratio of $31\% \pm 30\%$ (relative). Specificity of the method was confirmed.

Characteristics for the analytical method used for validation of boscalid residues in artificial soil

Specificity	The signal of the detected substance was overlapping with the matrix signal of the matrix blank samples under the experimental conditions. The peak area on the chromatogram of LOQ level sample is equal to 2444. The peak area on the matrix blank chromatograms is equal to 580 and 564 which is 23.7% and 23.1% of LOQ level, respectively. Matrix blank signal was not higher than 30% of the LOQ, which is acceptable. Specificity was verified using the ion transition ratio of $31\% \pm 30\%$ (relative). Specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 188.3 C + 308$ Coefficient of determination R^2 : 0.9966

	Number of data points: 6
Calibration range	Linearity range: 3 – 120 µg/L Range of analytical method: 43 µg/kg – 514.5 mg/kg
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 43 µg/kg LOD: 13 µg/kg

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance boscalid in artificial soil.

A 1.1.1.3 Description of analytical methods used in residue studies

A 1.1.1.3.1 HPLC-MS/MS (in wheat (seeds, whole plant, straw))

A 1.1.1.3.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/09

Report Magnitude of the residue of Boscalid (188425-85-6) in wheat (Raw Agricultural Commodity – RAC) grown in open field conditions after one application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe, Sala A., 2023, report no. LBN-0118-2023

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The analytical method for boscalid in wheat (seeds, whole plant, straw) was fully validated in this study according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).

Boscalid was determined by High-Performance Liquid Chromatography, tandem Mass Spectrometry (HPLC-MS/MS).

Chromatographic conditions

- Instrument: Agilent HPLC 1290 Infinity II coupled with Agilent MS spectrometer 6470A Triple Quadrupole
- Column: Acquity HSS PFP 2.1x150 mm (1.8 µm)
- Column temperature: 40 °C
- Mobile phase A: LC-MS grade water + 0.5% v/v formic acid
- Mobile phase B: LC-MS grade acetonitrile + 0.5% v/v formic acid

- Flow: 0.300 mL/min
- Elution gradient:
 - 0.00 min: 70 90 % - B 30 %
 - 0.5. min: A 70 % - B 30 %
 - 4.00 min: A 5 % - B 95 %
 - 6.00 min: A 5 % - B 95 %
 - 6.01 min: A 70 % - B 30 %
 - 8.50 min: A 70 % - B 30 %
- Injection volume: 1.00 µL
- Source type: electrospray ionisation (ESI) positive ionization mode
- Gas temperature: 300 °C
- Gas flow: 12 L/min
- Nebulizer: 60 psi
- Sheat Gas Heater: 400 °C
- Sheat gas floe: 12 L/min
- Capillary: 3000 V
- VCharging 1500 V
- Acquisition mode: multi-reaction monitoring (MRM) – positive polarity
- Boscalid retention time: 4.7 minutes (5.1 minutes – pods samples)
- Monitored transitions:

Analyte	Retention time (approx., min)	Detection ¹	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
Boscalid	4.7	Primary	343.0	271.0	110	35
		Confirmatory		272.0		

¹ Quantification was performed using the primary detection

Sample extraction

5 g (seeds and whole plant) or 2.5 g (straw) aliquot of each specimen were taken from the homogenised frozen sample and placed in a 50 mL screw capped centrifuge PE test tube. For recovery tests the sample was spiked at this stage.

10 mL of water (5 mL for whole plant samples) and 20 mL of acetonitrile were added to the sample and the mixture was vigorously shaken for 5 minutes A packet of QuEChERS salts (4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate, 0.5 disodium hydrogen citrate sesquihydrate) was added to the sample, the sample was shaken again for 1 minute and then centrifuged 5 minutes at 5000 rpm to allow phase separation.

The organic upper part was transferred in a HPLC glass vial e analysed by a HPLC-MS/MS system.

All the extracts were kept in refrigerated conditions and analysed within 24 hours from preparation. In any case the boscalid stability in the final extracts was verified after 3 days storage at 5 ± 3°C in dark conditions.

Samples were stored in frozen conditions (at ≤ -18°C) from sampling to analysis. The maximum freezer storage periods from sampling until extraction/analysis of the field samples are reported for each commodity in the following table.

Analyte	Commodity	Maximum freezer storage period between sampling and analysis ¹
Boscalid	Wheat Seeds	60
	Wheat Straw	65
	Wheat whole plant	119

¹ A storage stability study in frozen condition (-18°C) is ongoing to demonstrate Boscalid stability in the samples for a period longer than maximum time elapsed between sampling and analyses. The storage stability study will last 5 months (150 days). The LabAnalysis storage stability study is coded LBN-0126-2023

Validation of analytical method

Linearity/calibration

Instrumental linearity of response was checked by a 5-points calibration curve (single injections) using matrix matched standard solutions, prepared as described in the previous paragraphs. The equation of the calibration curve was calculated with the least square linear regression method (1/x weighed).

The absolute residuals in the residual plots are always randomly distributed confirming the linearity of response for each matrix analysed for both primary and confirmatory detection in compliance with SAN-TE/2020/12830, Rev.2.

The suitability of the chosen function was demonstrated by a residual analysis: the regression residual d_i describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - \hat{y}_i$$

where:

y_i is the measured value i ;

\hat{y}_i is the estimated value which corresponds to y_i and is derived from the calibration function.

A residual plot was obtained and a visual inspection was applied to decide if d_i were randomly distributed, thus demonstrating the linear calibration function.

Primary detection - Seeds

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 92.4977x + 31.7866$

Correlation factor $R^2 = 0.9984$

Confirmatory Detection – Seeds

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 90.1764x + 20.2001$

Correlation factor $R^2 = 0.9978$

Primary Detection - Straw

Calibration range: 0.25 – 25.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 58.2396x + 23.7705$

Correlation factor $R^2 = 0.9996$

Confirmatory Detection - Straw

Calibration range: 0.25 – 25.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 55.8241x + 26.8956$

Correlation factor $R^2 = 0.9996$

Primary Detection – Whole plant

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 65.5499x + 18.9286$

Correlation factor $R^2 = 0.9998$

Confirmatory Detection – Whole plant

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 63.7951x + 16.1613$

Correlation factor $R^2 = 0.9999$

Selectivity and specificity

These parameters were evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by the matrix.

Using a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: a blank sample, a fortified sample at LOQ level and a reference solution at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals higher 30 % of the LOQ level were detected in the untreated samples, these results are in compliance with the guideline requirements. The method was found to be selective for the determination of boscalid in wheat samples for

both primary and confirmatory MS/MS transitions.

Accuracy and precision

Accuracy and precision were verified by means of recovery tests carried out at the following spiking levels:

- 0.01 mg/kg – corresponding to the target LOQ - 5 replicates;
- 0.1 mg/kg – corresponding to 10xLOQ - 5 replicates.

The accuracy and precision results obtained are in compliance with SANTE/2020/12830 rev.2 requirements:

LOQ level (0.01 mg/kg)

Mean recovery 60 – 120%

RSD ≤30%

10xLOQ level (0.1 mg/kg)

Mean recovery 70 – 120%

RSD ≤20%

Primary transition:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Whole plant	23-118-01	Untreated	0	0	--	--	--	--
	23-118-02	Untreated	0	0	--	--	--	--
	23-118-03	LOQ 0.01 mg/kg	2.34	0.0091	93.6	91.7	3.5	3.8
	23-118-04		2.40	0.0097	96.2			
	23-118-05		2.18	0.0088	87.4			
	23-118-06		2.23	0.0087	89.4			
	23-118-07		2.30	0.0092	91.9			
	23-118-08	10xLOQ 0.1 mg/kg	22.5	0.0875	89.9	92.6	2.7	2.9
	23-118-09		23.9	0.0967	95.7			
	23-118-10		23.4	0.0927	93.6			
	23-118-11		22.4	0.0871	89.6			
	23-118-12		23.5	0.0953	93.9			
					Overall	92.1	3.0	3.2

Primary transition:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Seeds	23-118-13	Untreated	0	0	--	--	--	--
	23-118-14	Untreated	0	0	--	--	--	--
	23-118-15	LOQ 0.01 mg/kg	2.62	0.0104	104.7	103.1	3.1	3.0
	23-118-16		2.60	0.0104	104.1			
	23-118-17		2.48	0.0099	99.2			
	23-118-18		2.52	0.0101	100.8			
	23-118-19		2.67	0.0105	106.9			
	23-118-20	10xLOQ 0.1 mg/kg	28.1	0.1117	112.4	112.7	2.0	1.8
	23-118-21		28.4	0.1117	113.7			
	23-118-22		27.4	0.1094	109.4			
	23-118-23		28.6	0.1139	114.4			
	23-118-24		28.4	0.1133	113.8			
					Overall	107.9	5.6	5.2

Primary transition:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Straw	23-118-25	Untreated	0	0	--	--	--	--
	23-118-26	Untreated	0	0	--	--	--	--
	23-118-27	LOQ 0.01 mg/kg	1.22	0.0097	97.4	107.5	6.8	6.3
	23-118-28		1.39	0.0109	111.0			
	23-118-29		1.40	0.0113	112.2			
	23-118-30		1.41	0.0113	113.2			
	23-118-31		1.30	0.0105	103.7			
	23-118-32	10xLOQ 0.1 mg/kg	13.3	0.1071	106.7	109.2	1.9	1.8
	23-118-33		13.6	0.1095	108.7			
	23-118-34		13.7	0.1095	109.9			
	23-118-35		14.0	0.1115	111.9			
	23-118-36		13.6	0.1077	109.0			
					Overall	108.3	4.8	4.4

Limit of quantification (LOQ)

The Limit of quantification (LOQ) is defined as the lowest concentration at which an acceptable recovery is obtained. The target LOQ for this study was set at 0.01 mg/kg for all matrices analysed. This level corresponds to the lowest concentration levels tested during the recovery tests. At this level, the mean recovery and precision fulfil SANTE/2020/12830, Rev.2 requirements.

Limit of detection (LOD)

The Limit of Detection (LOD) is the lowest concentration tested at which the analyte produces an instrumental signal at least 3 times higher than the background noise of the chromatogram. It should be not higher than 30% of the LOQ value. The lowest concentrated standard injected can be considered the limit of detection.

This level corresponds is for all matrices analysed lower than 30% of the target LOQ (0.01 mg/kg).

In the following table the LOD values and the signal/noise (S/N) ratio for both primary and confirmatory detection are reported:

Matrix	Detection	LOD Concentration	S/N at LOD level
Wheat Seeds	343.0 → 271.0 (primary)	0.002 mg /kg (20% of LOQ)	3.0
	343.0 → 272.0 (confirmatory)		57.0
Wheat Straw	343.0 → 271.0 (primary)		12.0
	343.0 → 272.0 (confirmatory)		20.4
Wheat whole plant	343.0 → 271.0 (primary)		25.0
	343.0 → 272.0 (confirmatory)		26.5

Matrix effects

Assessments of matrix effect was performed by comparing the instrumental response (primary detection) of the same analytical standard: 50 µg/L - or 25 µg/L for pods samples (calibration standard L5) prepared both in solvent and matrix matched.

The result of the matrix effect evaluation are reported in the following table.

Matrix	Analyte	Concentration (µg/L)	Solvent	Peak area L5 stand-ard in solvent	Peak area L5 matrix matched	% Area in blank matrix /Area in Solvent	Matrix Effect (%)
Seeds	Boscalid	50	Acetonitrile	5742	4032	70.2	-29.8

Straw		25		3024	1793	59.3	-40.7
Whole plant		50		5742	2507	43.7	-56.3

According to SANTE/2020/12830 Rev.2 the matrix effect resulted relevant ($>\pm 20$) in all matrices analysed.

In order to nullify the matrix effect the calibration curves were always prepared using matrix matched standards.

Confirmatory

A simultaneous confirmation to the primary detection was performed using HPLC-MS/MS, monitoring an additional MS/MS transition. The confirmatory MS/MS transitions selected was 343.0 \rightarrow 272.0 m/z.

Confirmatory transition:					343.0 → 272.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Whole Plant	23-118-01	Untreated	0	0	--	--	--	--
	23-118-02	Untreated	0	0	--	--	--	--
	23-118-03	LOQ 0.01 mg/kg	2.49	0.0096	99.4	95.2	4.5	4.7
	23-118-04		2.49	0.0100	99.6			
	23-118-05		2.39	0.0097	95.5			
	23-118-06		2.25	0.0087	89.9			
	23-118-07		2.29	0.0091	91.4			
	23-118-08	10xLOQ 0.1 mg/kg	22.5	0.0877	90.2	91.7	4.1	4.4
	23-118-09		24.7	0.0998	98.8			
	23-118-10		22.1	0.0874	88.3			
	23-118-11		22.7	0.0884	90.9			
	23-118-12		22.6	0.0918	90.5			
Overall					93.4	4.4	4.7	

Confirmatory transition:					343.0 → 272.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Seeds	23-118-13	Untreated	0	0	--	--	--	--
	23-118-14	Untreated	0	0	--	--	--	--
	23-118-15	LOQ 0.01 mg/kg	2.70	0.0107	107.9	107.3	3.4	3.2
	23-118-16		2.68	0.0107	107.0			
	23-118-17		2.58	0.0103	103.0			
	23-118-18		2.81	0.0112	112.5			
	23-118-19		2.66	0.0105	106.2			
	23-118-20	10xLOQ 0.1 mg/kg	28.4	0.1128	113.5	111.4	2.5	2.2
	23-118-21		26.9	0.1058	107.8			
	23-118-22		27.7	0.1107	110.7			
	23-118-23		28.5	0.1134	113.9			
	23-118-24		27.8	0.1107	111.2			
					Overall	109.4	3.5	3.2

Confirmatory transition:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Straw	23-118-25	Untreated	0	0	--	--	--	--
	23-118-26	Untreated	0	0	--	--	--	--
	23-118-27	LOQ 0.01 mg/kg	1.15	0.0092	92.3	104.2	7.6	7.3
	23-118-28		1.27	0.0100	101.4			
	23-118-29		1.33	0.0107	106.5			
	23-118-30		1.39	0.0111	111.0			
	23-118-31		1.37	0.0111	109.7			
	23-118-32	10xLOQ 0.1 mg/kg	14.5	0.1163	115.8	112.8	2.3	2.0
	23-118-33		14.3	0.1154	114.5			
	23-118-34		14.0	0.1116	112.0			
	23-118-35		13.9	0.1106	111.0			
	23-118-36		13.8	0.1093	110.6			
	Overall					108.5	7.0	6.4

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability in sample extract

In order to check the stability of the analyte in the final extracts, aliquots of untreated sample extracts were spiked with known amounts of analyte at L5 calibration level). The stability in the extracts was tested for a period of 3 days (for Seeds and Whole Plant) and 5 days (for Straw) at $5 \pm 3^\circ\text{C}$ in dark conditions: after this period, the stored fortified extracts were analysed concurrently with the same matrix-matched standard solutions freshly prepared, used as reference. The measured instrumental responses were compared and the stability was expressed as the percentage ratio between the responses of the spiked extracts analysed after 3 days and 4 days, and the freshly spiked ones.

Matrix	Analyte	Analyte area Freshly pre-prepared standard	Analyte area Extract stored 3 days at $5 \pm 3^\circ\text{C}$	% Residual analyte (Stored/Fresh)	$\Delta\%$
Seeds	Boscalid	1376	1287	93.5	-6.5
Straw		5796	5700	98.3	-1.7
Whole plant		1128	982	87.1	-12.9

The stability of the analyte in the extract can be considered proven for 3 days and 5 days at $5 \pm 3^\circ\text{C}$ in the dark since the recovery of the stored spiked sample is within the range of 70-120% measured against the freshly prepared one, as required by the SANTE/2020/12830 rev.2 guidance document.

Stock solution stability

The stock solution stability was proven after 17 days storage at $5 \pm 3^\circ\text{C}$ (storage from 19/09/2023 to 06/10/2023).

The stock solution stability was verified comparing on 06/10/2023 the instrumental response (UV absorbance at 230 nm) of a standard diluted solution of boscalid (100 mg/L) obtained using the stock solution freshly prepared on 06/10/2023 with the instrumental response of the same standard diluted solution (100 mg/L) prepared using aged stock solution of boscalid prepared on 19/09/2023 and stored refrigerated for 17 days in the dark at $5 \pm 3^\circ\text{C}$. 5 replicate injections for both standard solutions were analysed.

The results obtained are reported in the table below.

Sample	Stock solution used	Instrumental response (primary detection)	Mean Instrumental response	Stock solution stability
Mix 100 mg/L	Stored 17 days at 5 ± 3°C	385.0	382.0	9.5%
		384.1		
		380.9		
		382.7		
		377.5		
Mix 100 mg/L	Freshly prepared	346.3	348.9	
		354.1		
		345.7		
		347.2		
		351.0		

The boscalid stock solution stored at $5 \pm 3^{\circ}\text{C}$ can be considered stable for at least 17 days: the instrumental response difference between stored and fresh stock solution resulted lower than 10% (in compliance with SANTE/2020/12830 rev.2 requirements).

Procedural recovery check

Procedural recovery tests at 3 spiking levels were carried out during the study on control sample aliquots to verify in all analytical session the method performances of the validated method.

Untreated samples aliquots of each matrix were spiked at the following spiking levels:

- Spiking level 0.01 mg/kg (nominal) – 4 replicates (LOQ)
- Spiking level 0.1 mg/kg (nominal) – 5 replicates (10xLOQ),
- Spiking level 10.0 mg/kg (nominal) – 4 replicates (1000xLOQ)

The spiked samples were then extracted and analysed according to the analytical method. The results of the recovery tests performed are reported in the table below.

Matrix	LabAnalysis code	Fortification Level (mg/kg) (nominal)	Recovery (%)
Seeds	CDS-23-1301 RC1	0.01 mg/kg (LOQ)	95.2
	CDS-23-1301 RC2	0.1 mg/kg (10xLOQ)	92.1
Straw	CDS-23-1316 RC1	0.01 mg/kg (LOQ)	70.4
	CDS-23-1316 RC2	0.1 mg/kg (10xLOQ)	76.9
	CDS-23-1316 RC3		70.1
	CDS-23-1316 RC4	10.0 mg/kg (1000xLOQ)	77.1
	CDS-23-1316 RC5		81.8
Whole Plant	CDS-23-1316 RC1	0.01 mg/kg (LOQ)	77.6
	CDS-23-1316 RC2		91.8
	CDS-23-1316 RC3	0.1 mg/kg (10xLOQ)	71.8
	CDS-23-1316 RC4		74.3
	CDS-23-1316 RC5	10.0 mg/kg (1000xLOQ)	90.0
	CDS-23-1316 RC6		85.7

Conclusion

Parameter	Result	SANTE/2020/12830 rev.2 limit				
Matrix effect	Grain: - 29.8 % / significant Straw: - 40.7 % / significant Whole Plant: - 56.3 % / significant	< ±20%				
Calibration (matrix-matched)	Range:0.002 – 0.2 mg/kg (from 20% of LOQ to 20xLOQ) The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.	At least from 30% of LOQ to at least 20% above the highest value measured Residuals randomly distributed				
Accuracy and precision	Seeds					
	Level	Concentration (mg/kg)	Primary Transition (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 271.0	103.1	3.0	
	10xLOQ (n = 5)	0.100		112.7	1.8	
	Overall (n = 10)	/		107.9	5.2	
	Level	Concentration (mg/kg)	Confirmatory Transition (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 272.0	107.3	3.2	
	10xLOQ (n = 5)	0.100		111.4	2.2	
	Overall (n = 10)	/		109.4	3.2	
	n = number of replicates					
	Straw					
	Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
LOQ (n = 5)	0.010	m/z 343.0 → 271.0	107.5	6.3		
10xLOQ (n = 5)	0.100		109.2	1.8		
Overall (n = 10)	/		108.3	4.4		
Level	Concentration (mg/kg)	Confirmatory Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD		
LOQ (n = 5)	0.010	m/z 343.0 → 272.0	104.2	7.3		
10xLOQ (n = 5)	0.100		112.8	2.0		
Overall (n = 10)	/		108.5	6.4		
n = number of replicates						
Whole Plant						
Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD		
LOQ (n = 5)	0.010	m/z 343.0 → 271.0	91.7	3.8		
10xLOQ (n = 5)	0.100		92.6	2.9		
Overall (n = 10)	/		92.1	3.2		
	Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 271.0	95.2	4.7	
	10xLOQ (n = 5)	0.100		91.7	4.4	
	Overall (n = 10)	/		93.4	4.7	
	n = number of replicates					
Limit of quantification (LOQ)	verified at 0.01 mg/kg – each matrix accuracy and precision data in compliance with SANTE/2020/12830 rev.2 requirements				LOQ: lowest validated level with sufficient recovery and precision	
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ, lowest calibration standard) (signal/noise ratio higher than 3 at this level)				LOD < 30% of LOQ	
Selectivity and specificity	Verified: no interferences found untreated samples in amounts higher than the 30% of the LOQ (< LOD)				Blank values not higher than 30% of LOQ	
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory MS/MS transition. Calibration data, recovery and precision in compliance with the requirements				Confirmation by monitoring at least 1 additional MS/MS transition, providing linearity, recovery, precision, selectivity	
Stability of the standard solution	Verified for 17 days at 5 ± 3°C in the dark (stock solution in acetonitrile): the difference between stored and fresh solution resulted : +9.5%				< 10%	
Stability of the analyte in the sample extract	After 3 days in the dark at 5 ± 3°C – % Recovery Stored/Fresh Seeds: - 6.5 % Whole Plant: - 1.7 % After 5 days in the dark at 5 ± 3°C – % Recovery Stored/Fresh Straw: -12.9%				70-120%	

The validation of the analytical method was carried out in compliance with SANTE/2020/12830 Rev.2 guidance document. The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of boscalid in wheat samples (seeds whole plant and straw) in the range 0.002 – 0.200 mg/kg.

A 1.1.1.3.2 HPLC-MS/MS (in rape (seeds, whole plant, plant without pods, pods))

A 1.1.1.3.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/10
Report	Magnitude of the residue of Boscalid (188425-85-6) in oilseed rape (Raw Agricultural Commodity – RAC) grown in open field conditions after two application of formulated product BSK-FUN 500 SC – four harvest and four decline curve trials in Northern Europe, Sala A., 2023, report no. LBN-0119-2023
Guideline(s):	SANTE/2020/12830 rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for boscalid in rape (seeds, whole plant, plant without pods, pods) was fully validated in this study according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).

Boscalid was determined by High-Performance Liquid Chromatography, tandem Mass Spectrometry (HPLC-MS/MS).

Chromatographic conditions

- Instrument: Agilent HPLC 1290 Infinity II coupled with Agilent MS spectrometer 6470A Triple Quadrupole
- Column: Acquity HSS PFP 2.1x150 mm (1.8 µm)
- Column temperature: 40°C
- Mobile phase A: LC-MS grade water + 0.5% v/v formic acid
- Mobile phase B: LC-MS grade acetonitrile + 0.5% v/v formic acid
- Flow: 0.300 mL/min
- Elution gradient:

Time (minutes)	A%	B%
All matrices except pods		
0.00	70	30
0.50	70	30
4.00	5	95
6.00	5	95
6.01	70	30
8.50	70	30
Pods		
0.00	70	30
0.50	70	30
5.00	5	95
10.00	5	95
10.01	70	30
12.50	70	30

- Injection volume: 1.00 µL
- Source type: electrospray ionisation (ESI) positive ionization mode
- Gas temperature: 300 °C
- Gas flow: 12 L/min
- Nebulizer: 60 psi
- Sheat Gas Heater: 400 °C
- Sheat gas floe: 12 L/min
- Capillary: 3000 V
- VCharging 1500 V
- Acquisition mode: multi-reaction monitoring (MRM) – positive polarity
- Boscalid retention time: 4.7 minutes (5.1 minutes – pods samples)
- Monitored transitions:

Analyte	Retention time (approx., min)	Detection ¹	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
Boscalid	4.7	Primary	343.0	271.0	110	35
		Confirmatory		272.0		

¹ Quantification was performed using the primary detection

Sample extraction

5 g (seeds and whole plant) or 2.5 g (pods) aliquot of each specimen were taken from the homogenised frozen sample and placed in a 50 mL screw capped centrifuge PE test tube. For recovery tests the sample was spiked at this stage.

10 mL of water (5 mL for whole plant samples) and 20 mL of acetonitrile were added to the sample and the mixture was vigorously shaken for 5 minutes. A packet of QuEChERS salts (4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate, 0.5 disodium hydrogen citrate sesquihydrate) was added to the sample, the sample was shaken again for 1 minute and then centrifuged 5 minutes at 5000 rpm to allow phase separation.

The organic upper part was transferred in a HPLC glass vial e analysed by a HPLC-MS/MS system.

All the extracts were kept in refrigerated conditions and analysed within 24 hours from preparation. In any case the boscalid stability in the final extracts was verified after 3 days storage (Seed and Whole plant extracts) or 4 days storage (Pods extracts) at 5 ± 3°C in dark conditions.

Samples were stored in frozen conditions (at ≤ -18°C) from sampling to analysis. The maximum freezer storage periods from sampling until extraction/analysis of the field samples are reported for each commodity in the following table.

Analyte	Commodity	Maximum freezer storage period between sampling and analysis ¹
Boscalid	Whole plant	113
	Plant without pods	88
	Pods	89
	Seeds	72

¹ A storage stability study in frozen condition (-18°C) is ongoing to demonstrate Boscalid stability in the samples for a period longer than maximum time elapsed between sampling and analyses. The storage stability study will last 5 months (150 days). The LabAnalysis storage stability study is coded LBN-0126-2023

Validation of analytical method

Linearity/calibration

Instrumental linearity of response was checked by a 5-points calibration curve (single injections) using matrix matched standard solutions, prepared as described in the previous paragraphs. The equation of the calibration curve was calculated with the least square linear regression method (1/x weighed). The absolute residuals in the residual plots are always randomly distributed confirming the linearity of response for each matrix analysed for both primary and confirmatory detection in compliance with SAN-TE/2020/12830, Rev.2.

The suitability of the chosen function was demonstrated by a residual analysis: the regression residual d_i describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - y_{yi}$$

where:

y_i is the measured value i ;

y_{yi} is the estimated value which corresponds to y_i and is derived from the calibration function.

A residual plot was obtained and a visual inspection was applied to decide if d_i were randomly distributed, thus demonstrating the linear calibration function.

Primary detection - Seeds

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 41.638611x + 16.596151$

Correlation factor $R^2 = 0.9993$

Confirmatory Detection – Seeds

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 40.004338x + 10.494044$

Correlation factor $R^2 = 0.9990$

Primary Detection - Pods

Calibration range: 0.25 – 25.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $20.777697x + 1.740426$

Correlation factor $R^2 = 0.9996$

Confirmatory Detection - Pods

Calibration range: 0.25 – 25.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $19.512552x + 1.367157$

Correlation factor $R^2 = 0.9996$

Primary Detection – Whole plant

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 59.688039x + 10.333632$

Correlation factor $R^2 = 0.9999$

Confirmatory Detection – Whole plant

Calibration range: 0.50 – 50.0 µg/L (0.002 – 0.200 mg/kg on sample – 20% LOQ – 20xLOQ)

Calibration curve equation: $y = 59.688039x + 10.333632$

Correlation factor $R^2 = 0.9999$

Selectivity and specificity

These parameters were evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by the matrix. Using a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: a blank sample, a fortified sample at LOQ level and a reference solution at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals higher 30 % of the LOQ level were detected in the untreated samples, these results are in compliance with the guideline requirements. The method was found to be selective for the determination of boscalid in oilseed rape samples for both primary and confirmatory MS/MS transitions.

Accuracy and precision

Accuracy and precision were verified by means of recovery tests carried out at the following spiking levels:

- 0.01 mg/kg – corresponding to the target LOQ - 5 replicates;

- 0.1 mg/kg – corresponding to 10xLOQ - 5 replicates.

The accuracy and precision results obtained are in compliance with SANTE/2020/12830 rev.2 requirements:

LOQ level (0.01 mg/kg)

Mean recovery 60 – 120%

RSD ≤30%

10xLOQ level (0.1 mg/kg)

Mean recovery 70 – 120%

RSD ≤20%

Primary transition:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Whole plant	23-119-01	Untreated	0	0	--	--	--	--
	23-119-02	Untreated	0	0	--	--	--	--
	23-119-03	LOQ 0.01 mg/kg	2.53	0.0099	101.1	97.0	3.9	4.0
	23-119-04		2.41	0.0095	96.5			
	23-119-05		2.36	0.0094	94.6			
	23-119-06		2.52	0.0099	100.7			
	23-119-07		2.30	0.0091	92.2			
	23-119-08	10xLOQ 0.1 mg/kg	23.1	0.0929	92.6	92.7	0.6	0.7
	23-119-09		23.3	0.0915	93.1			
	23-119-10		23.0	0.0915	91.9			
	23-119-11		23.4	0.0923	93.4			
	23-119-12		23.1	0.0931	92.3			
					Overall	94.8	3.5	3.7

Primary detection:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Pods	23-119-13	Untreated	0	0	--	--	--	--
	23-119-14	Untreated	0	0	--	--	--	--
	23-119-15	LOQ 0.01 mg/kg	1.23	0.0096	98.0	105.2	8.1	7.7
	23-119-16		1.48	0.0116	118.4			
	23-119-17		1.34	0.0103	107.1			
	23-119-18		1.25	0.0100	100.1			
	23-119-19		1.28	0.0100	102.3			
	23-119-20	10xLOQ 0.1 mg/kg	10.7	0.0859	85.6	89.4	3.2	3.6
	23-119-21		11.6	0.0936	92.5			
	23-119-22		11.6	0.0923	93.0			
	23-119-23		11.1	0.0882	88.5			
	23-119-24		11.0	0.0869	87.6			
					Overall	97.3	10.1	10.4

Primary detection:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Seeds	23-119-25	Untreated	0	0	--	--	--	--
	23-119-26	Untreated	0	0	--	--	--	--
	23-119-27	LOQ 0.01 mg/kg	2.41	0.0096	96.3	93.9	6.2	6.6
	23-119-28		2.40	0.0097	96.1			
	23-119-29		2.54	0.0102	101.8			
	23-119-30		2.16	0.0085	86.4			
	23-119-31		2.23	0.0089	89.0			
	23-119-32	10xLOQ 0.1 mg/kg	23.7	0.0945	94.9	93.1	1.3	1.4
	23-119-33		23.2	0.0916	92.7			
	23-119-34		22.8	0.0911	91.3			
	23-119-35		23.4	0.0934	93.5			
	23-119-36		23.3	0.0923	93.2			
						Overall	93.5	4.2

Limit of quantification (LOQ)

The Limit of quantification (LOQ) is defined as the lowest concentration at which an acceptable recovery is obtained. The target LOQ for this study was set at 0.01 mg/kg for all matrices analysed. This level corresponds to the lowest concentration levels tested during the recovery tests. At this level, the mean recovery and precision fulfil SANTE/2020/12830, Rev.2 requirements.

Limit of detection (LOD)

The Limit of Detection (LOD) is the lowest concentration tested at which the analyte produces an instrumental signal at least 3 times higher than the background noise of the chromatogram. It should be not higher than 30% of the LOQ value. The lowest concentrated standard injected can be considered the limit of detection. This level corresponds is for all matrices analysed lower than 30% of the target LOQ (0.01 mg/kg). In the following table the LOD values and the signal/noise (S/N) ratio for both primary and confirmatory detection are reported:

Matrix	Detection	LOD Concentration	S/N at LOD level
Whole plant	343.0 → 271.0 (primary)	0.002 mg /kg (20% of LOQ)	3
	343.0 → 272.0 (confirmatory)		31
Pods	343.0 → 271.0 (primary)		8
	343.0 → 272.0 (confirmatory)		3
Seeds	343.0 → 271.0 (primary)		21
	343.0 → 272.0 (confirmatory)		17

Matrix effects

Assessments of matrix effect was performed by comparing the instrumental response (primary detection) of the same analytical standard: 50 µg/L - or 25 µg/L for pods samples (calibration standard L5) prepared both in solvent and matrix matched.

The result of the matrix effect evaluation are reported in the following table.

Matrix	Analyte	Concentration (µg/L)	Peak area L5 standard in solvent (acetonitrile)	Peak area L5 matrix matched	% Area in blank matrix /Area in Solvent	Matrix Effect (%)
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Whole plant	Boscalid	50	6025	4237	70.3	-29.7
Pods		25	3010	2529	84.0	-16.0
Seeds		50	6025	1919	31.9	-68.1

According to SANTE/2020/12830 Rev.2 the matrix effect is relevant ($>\pm 20$) for whole plant and seeds samples and not relevant for pods samples.

In order to nullify the matrix effect the calibration curves were always prepared using matrix matched standards.

Confirmatory

A simultaneous confirmation to the primary detection was performed using HPLC-MS/MS, monitoring an additional MS/MS transition. The confirmatory MS/MS transitions selected was 343.0 → 272.0 m/z.

Confirmatory detection:					343.0 → 272.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Whole Plant	23-119-01	Untreated	0	0	--	--	--	--
	23-119-02	Untreated	0	0	--	--	--	--
	23-119-03	LOQ 0.01 mg/kg	2.60	0.0102	104.1	101.0	5.0	4.9
	23-119-04		2.41	0.0095	96.5			
	23-119-05		2.65	0.0106	106.2			
	23-119-06		2.58	0.0102	103.2			
	23-119-07		2.37	0.0094	94.9			
	23-119-08	10xLOQ 0.1 mg/kg	24.7	0.0991	98.7	96.9	2.0	2.0
	23-119-09		23.8	0.0935	95.1			
	23-119-10		24.4	0.0972	97.6			
	23-119-11		24.7	0.0975	98.7			
	23-119-12		23.6	0.0953	94.6			
Overall					99.0	4.2	4.2	

Confirmatory detection:					343.0 → 272.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Pods	23-119-13	Untreated	0	0	--	--	--	--
	23-119-14	Untreated	0	0	--	--	--	--
	23-119-15	LOQ 0.01 mg/kg	1.16	0.0090	92.6	100.1	8.5	8.4
	23-119-16		1.42	0.0111	113.4			
	23-119-17		1.29	0.0099	103.0			
	23-119-18		1.18	0.0094	94.0			
	23-119-19		1.22	0.0095	97.4			
	23-119-20	10xLOQ 0.1 mg/kg	11.1	0.0891	88.7	94.6	6.0	6.4
	23-119-21		11.2	0.0911	90.0			
	23-119-22		12.5	0.0993	100.1			
	23-119-23		12.8	0.1016	102.0			
	23-119-24		11.5	0.0916	92.3			
Overall					97.3	7.5	7.7	

Confirmatory detection:					343.0 → 271.0			
Matrix	Analytical code	Spiking level	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)	Mean Recovery (%)	SD	% RSD
Seeds	23-119-25	Untreated	0	0	--	--	--	--
	23-119-26	Untreated	0	0	--	--	--	--
	23-119-27	LOQ 0.01 mg/kg	2.04	0.0082	81.6	90.5	8.8	9.7
	23-119-28		2.30	0.0093	92.2			
	23-119-29		2.48	0.0099	99.1			
	23-119-30		2.03	0.0080	81.0			
	23-119-31		2.47	0.0099	98.7			
	23-119-32	10xLOQ 0.1 mg/kg	23.3	0.0927	93.0	91.6	2.3	2.5
	23-119-33		22.8	0.0900	91.1			
	23-119-34		22.5	0.0897	89.9			
	23-119-35		23.7	0.0945	94.7			
	23-119-36		22.3	0.0882	89.1			
						Overall	91.0	6.1

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability in sample extract

In order to check the stability of the analyte in the final extracts, aliquots of untreated sample extracts were spiked with known amounts of analyte at L5 calibration level). The stability in the extracts was tested for a period of 3 days (for Seeds and Whole Plant) and 4 days (for Pods) at $5 \pm 3^\circ\text{C}$ in dark conditions: after this period, the stored fortified extracts were analysed concurrently with the same matrix-matched standard solutions freshly prepared, used as reference. The measured instrumental responses were compared and the stability was expressed as the percentage ratio between the responses of the spiked extracts analysed after 3 days and 4 days, and the freshly spiked ones.

Matrix	Analyte	Analyte area Freshly pre-prepared standard	Analyte area Extract stored 3 days at $5 \pm 3^\circ\text{C}$	% Residual analyte (Stored/Fresh)	$\Delta\%$
Seeds	Boscalid	1979	1899	96.0	-4.0
Whole plant		6846	6247	91.3	-8.7
Pods		1684	1360	80.8	-19.2

The stability of the analyte in the extract can be considered proven for 3 days and 4 days at $5 \pm 3^\circ\text{C}$ in the dark since the recovery of the stored spiked sample is within the range of 70-120% measured against the freshly prepared one, as required by the SANTE/2020/12830 rev.2 guideline.

Stock solution stability

The stock solution stability in refrigerated conditions ($5 \pm 3^\circ\text{C}$) was verified for a period longer than maximum time elapsed between stock solution preparation and its last use (stability tested after 17 days storage: 19/09/2023 – 06/10/2023) in the concurrent validation study coded LBN-0118-2023, Magnitude of the residue of Boscalid (188425-85-6) in wheat (Raw Agricultural Commodity – RAC) grow in open field conditions after one application of a formulated product BSK-FUN 500 SC – four harvest and four de-

cline curve trials in Northern Europe, Test Facility LabAnalysis, Study director Alberto Sala.

On study LBN-118-2023 the stock solution stability was verified comparing on 06/10/2023 the instrumental response (UV absorbance at 230 nm) of a standard diluted solution of Boscalid (100 mg/L) obtained using the Stock solution freshly prepared on 06/10/2023 with the instrumental response of the same standard diluted solution (100 mg/L) prepared using aged stock solution of Boscalid prepared on 19/09/2023 and stored refrigerated for 17 days in the dark at $5 \pm 3^\circ\text{C}$. 5 replicate injections for both standard solutions were analysed.

The results obtained (data acquired on LBN-0118-2023) are reported in the table below.

Sample	Stock solution used	Instrumental response (primary detection)	Mean Instrumental response	Stock solution stability
Mix 100 mg/L	Stored 17 days at 5 ± 3°C	385.0	382.0	9.5%
		384.1		
		380.9		
		382.7		
		377.5		
Mix 100 mg/L	Freshly prepared	346.3	348.9	
		354.1		
		345.7		
		347.2		
		351.0		

The boscalid stock solution stored at $5 \pm 3^\circ\text{C}$ can be considered stable for at least 17 days: the instrumental response difference between stored and fresh stock solution resulted lower than 10% (in compliance with SANTE/2020/12830 rev.2 requirements).

Procedural recovery check

Procedural recovery tests at 3 spiking levels were carried out during the study on control sample aliquots to verify in all analytical session the method performances of the validated method.

Untreated samples aliquots of each matrix were spiked at the following spiking levels:

- Spiking level 0.01 mg/kg (nominal) – 3 replicates (LOQ)
- Spiking level 0.1 mg/kg (nominal) – 2 replicates (10xLOQ),
- Spiking level 10.0 mg/kg (nominal) – 5 replicates (1000xLOQ)

After spiking step, the samples were prepared according to the procedure described in the previous paragraphs. The fortification procedure is summarised in the table below.

Matrix	LabAnalysis code	Fortification Level (mg/kg)	Recovery (%)
Seeds	CDS-23-1364 RC1	0.01 mg/kg (LOQ)	92.6
	CDS-23-1364 RC2	0.1 mg/kg (10xLOQ)	90.6
Pods	CDS-23-1598 RC1	0.01 mg/kg (LOQ)	98.5
	CDS-23-1598 RC2	10 mg/kg (1000xLOQ)	83.6
	CDS-23-1598 RC4		90.8
Whole Plant	CDS-23-1597 RC1	0.01 mg/kg (LOQ)	80.1
	CDS-23-1597 RC2	0.1 mg/kg (10xLOQ)	119.2
	CDS-23-1597 RC3	10 mg/kg (1000xLOQ)	70.4
	CDS-23-1597 RC4		75.0
	CDS-23-1597 RC5		88.6

Conclusion

Parameter	Result	SANTE/2020/12830 rev.2 limit				
Matrix effect	Seeds: - 68.1 % / significant Pods: - 16.0 % / not significant Whole Plant: - 29.7 % / significant	< ±20%				
Calibration (matrix-matched)	Range: 0.002 – 0.2 mg/kg (from 20% of LOQ to 20xLOQ)	At least from 30% of LOQ to at least 20% above the highest value measured Residuals randomly distributed				
	The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration.					
Accuracy and precision	Seeds					
	Level	Concentration (mg/kg)	Primary Transition (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 271.0	93.9	6.6	
	10xLOQ (n = 5)	0.100		93.1	1.4	
	Overall (n = 10)	/		93.5	4.5	
	Level	Concentration (mg/kg)	Confirmatory Transition (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 272.0	90.5	9.7	
	10xLOQ (n = 5)	0.100		91.6	2.5	
	Overall (n = 10)	/		91.0	6.7	
	n = number of replicates					
	Pods					
	Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 271.0	105.2	7.7	
	10xLOQ (n = 5)	0.100		89.4	3.6	
	Overall (n = 10)	/		97.3	10.4	
	Level	Concentration (mg/kg)	Confirmatory Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD	
	LOQ (n = 5)	0.010	m/z 343.0 → 272.0	100.1	8.4	
	10xLOQ (n = 5)	0.100		94.6	6.4	
	Overall (n = 10)	/		97.3	7.7	
	n = number of replicates					
Whole Plant						
Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD		
LOQ (n = 5)	0.010	m/z 343.0 → 271.0	97.0	4.0		
10xLOQ (n = 5)	0.100		92.7	0.7		
Overall (n = 10)	/		94.8	3.7		
Level	Concentration (mg/kg)	Primary Detection (MS/MS positive)	Mean Recovery (%)	Precision % RSD		
LOQ (n = 5)	0.010	m/z 343.0 → 271.0	101.0	4.9		
10xLOQ (n = 5)	0.100		96.9	2.0		
Overall (n = 10)	/		99.0	4.2		
n = number of replicates						
Limit of quantification (LOQ)	verified at 0.01 mg/kg – each matrix accuracy and precision data in compliance with SANTE/2020/12830 rev.2 requirements					LOQ: lowest validated level with sufficient recovery and precision
Limit of detection (LOD)	verified at 0.002 mg/kg (20% of LOQ, lowest calibration standard) (signal/noise ratio higher than 3 at this level)					LOD < 30% of LOQ
Selectivity and specificity	Verified: no interferences found untreated samples in amounts higher than the 30% of the LOQ (< LOD)					Blank values not higher than 30% of LOQ
Confirmation	Confirmation achieved by simultaneous determination of a confirmatory MS/MS transition. Calibration data, recovery and precision in compliance with the requirements					Confirmation by monitoring at least 1 additional MS/MS transition, providing linearity, recovery, precision, selectivity
Stability of the standard solution	Verified for 17 days at 5 ± 3°C in the dark (stock solution in acetonitrile): the difference between stored and fresh solution resulted: 9.5%					< 10%
Stability of the analyte in the sample extract	After 3 days in the dark at 5 ± 3°C – % Recovery Stored/Fresh Seeds: - 4.0 % Whole Plant: -8.7 % After 4 days in the dark at 5 ± 3°C – % Recovery Stored/Fresh Pods: -19.2%					70-120%

The validation of the analytical method was carried out in compliance with SANTE/2020/12830 Rev.2

guidance document. The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of boscalid in rape samples (seeds, whole plant, plant without pods, pods) in the range 0.002 – 0.200 mg/kg.

A 1.1.1.3.3 LC-MS/MS (in honey)

A 1.1.1.3.3.1 Method validation

Comments of zRMS: Method is accepted

Reference: KCP 5.1.2/12 and KCP 5.2/01

Report Validation of the Analytical Method for the Analysis of boscalid in Honey, Schlewitz P., 2023, report no. R C3128

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to validate the analytical method for the analysis of boscalid in honey. Residues are extracted from honey with water and acetonitrile/acetic acid 99.9/0.1 % mixture. After addition of magnesium sulphate and sodium chloride, the mixture is shaken intensively and centrifuged for phase separation. An aliquot of the organic phase is filtered through PET filter, transferred into a vial for LC-MS/MS analysis.

The method was validated at the limit of quantification of 0.01 mg/kg.

The analytical method was validated according to SANTE/2020/12830, Rev.2.

The following points were examined during the study: matrix effects, calibration, limit of detection (LOD), limit of quantification (LOQ), recovery and repeatability, selectivity and specificity, confirmation, stability results for extracts, stability results for matrix-matched standard solutions.

Chromatographic conditions

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1 x 50 mm	Supplier reference	186002350	Comment	-
Development Column ANADIAG Number	334	Stationary Phase	C18		

Mobile phase

A =	Water + 0.1% formic acid
B =	Acetonitrile + 0.1% formic acid
Sample temperature	15°C
Column temperature	40°C

Elution

Elution	Time min	Flow mL/min	Composition (%)		Curve* (type)	Elution	Time min	Flow mL/min	Composition (%)		Curve* (type)
			A	B					A	B	
Pg1	0.00	0.4	90	10	-	Pg5	4.00	0.4	90	10	6
Pg2	2.00	0.4	0	100	6	Pg6	-	-	-	-	-
Pg3	3.00	0.4	0	100	6	Pg7	-	-	-	-	-
Pg4	3.25	0.4	90	10	6	Pg8	-	-	-	-	-

*6=linear

Detector

IONISATION mode	ES <input checked="" type="checkbox"/>	APCI <input type="checkbox"/>	Source temperature (°C)	150
Polarity	Pos <input checked="" type="checkbox"/>	Neg <input type="checkbox"/>	Desolvation temperature (°C)	650
Capillary (kV) ES	3		Cone gas Flow (L/Hr)	50
APCI current Corona (µA)	-		Desolvation Gas Flow (L/Hr)	500

Analyte	Cone voltage (V)	Collision voltage (V)	Dwell time (ms)	TRANSITION	RT (min.)
				Parent > Daughter	
Boscalid	30	20	80	343.1 > 307.0 **	≈ 1.8
	30	35	80	343.1 > 271.0 ***	
	30	20	80	343.1 > 139.9	

** Used for quantification / *** Used for qualification

Validation results

Matrix effects

Assessment of matrix effects was performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix for both primary and confirmatory methods.

Matrix effects, expressed in % enhancement or suppression were evaluated according to the following equation:

$$\text{Recovery for matrix effect (\%)} = 100 \times \frac{\text{FRE (matrix)}}{\text{FR (solvent)}} - 100$$

FRE (matrix) = average response factor (matrix)

FR (solvent) = response factor (solvent)

Matrix effects are considered significant if they exceed ±20%.

Matrix	Analyte	Concentration (ng/mL)	Matrix effect (%)
Matrix effects – Primary method			
Honey	Boscalid	25	-20.5%
Matrix effects – Confirmatory method			
Honey	Boscalid	25	-22.5%

Matrix effects (enhancement or suppression) on the instrument response were considered significant. Thus, matrix-matched calibration solutions were used for calibration.

Calibration

The analytical calibration consisted of matrix-matched calibration solutions of boscalid, at least at 5 concentration levels, ranged from 0.77 ng/mL to 30.5 ng/mL (corresponding to 0.003 to 0.12 mg/kg).

The calibration covered two orders of magnitude and ranged from 30% of the LOQ to 20% above the highest level. Standard concentrations were distributed evenly over the full calibration range.

Calibration curves were run for each analysis sequence for both primary and confirmatory methods.

An example of a typical calibration plot, the equation of the calibration line, the linear correlation coefficient and the regression residuals plot is given for both primary and confirmatory methods.

The linear correlation coefficients were > 0.990, showing good linearity with regression residuals randomly distributed for both primary and confirmatory methods.

Limit of detection

The limit of detection (LOD) is expressed as lowest calibration standard.
The LOD was 0.77 ng/mL for boscalid in honey (corresponding to 0.003 mg/kg).

Limit of quantification

The limit of quantification (LOQ) is the lowest validated level with sufficient recovery and precision.
The LOQ was 0.01 mg/kg for boscalid in honey.

Recovery and repeatability

Untreated samples

Two untreated samples were extracted concurrently with fortified samples. Residue levels were reported below. No interferences above 30% of the limit of quantification were recorded.

Fortified samples – primary method

For the primary method, recovery and repeatability (as precision, % RSD) tests were performed by untreated control samples spiked with boscalid before extraction at the following fortification levels:

- LOQ (5 samples),
- 10 x LOQ (5 samples).

Fortified samples – confirmatory method

For the confirmatory method, recovery and repeatability (as precision, % RSD) tests were performed by untreated control samples spiked with boscalid at the LOQ (5 samples) before extraction.

Analyte	Matrix	Fortifica- tion level (mg/kg)	Mean re- coveries (%)	RSD (%)	Min recov- ery (%)	Max re- covery (%)	Number of fortified samples (n)
Primary method							
Boscalid	Honey	0.01	82.6	3.0	78.5	85.2	5
		0.10	77.7	4.4	73.9	82.6	5
Confirmatory method							
Boscalid	Honey	0.01	85.8	3.3	81.0	88.0	5

For the primary method, recovery and repeatability (as precision, % RSD) data complied with the requirements of the SANTE/2020/12830, Rev.2 guideline as mean recoveries were within the range 70-120% with RSD less than 20% for spiked samples at 0.01 mg/kg and for spiked samples at 0.10 mg/kg.

For the confirmatory method, recovery and repeatability (as precision, % RSD) data complied with the requirements of the SANTE/2020/12830, Rev.2 guideline as mean recoveries were within the range 70-120% and RSD were less than 20% for spiked samples at the LOQ level.

Selectivity and specificity

Representative, clearly labelled chromatograms of standard at the lowest calibrated level, untreated sample and sample fortified at the lowest fortification level for both primary and confirmatory methods were provided to prove selectivity of the method.

Mass spectrum was provided to justify the selection of ions used for determination.

Untreated samples (non-fortified samples) were determined from the matrix used in fortification experiments and the boscalid residues were not higher than 30% of the LOQ for both primary and confirmatory methods.

Confirmation

The confirmatory method was required to confirm that the primary method detected the correct analyte (analyte identity) and that the analyte signal of the primary method was quantitatively correct and not affected by any other compound.

Confirmation simultaneously to primary detection:

The confirmatory method was achieved by monitoring 1 additional transition.

Primary transition: m/z 343.1 > 307.0

Confirmatory transition: m/z 343.1 > 271.0

The following validation data were provided for the confirmatory method: matrix effects, calibration data, recovery and precision data for 5 samples fortified at the LOQ and for 2 untreated samples and selectivity/specificity.

Storage stability of extracts and standard solutions

The storage stability of the analyte in extracts was evaluated by analysing spiked samples extracts after frozen storage.

The frozen storage stability of standard solutions was evaluated by comparing response factors obtained for stored solutions to freshly prepared solutions.

Storage stability of extracts

Spiked samples at 10xLOQ level were stored frozen after samples extraction and analysed against freshly prepared standards to check the stability of the final extracts.

The stability of the analyte in the final extracts was sufficiently proven according to the SANTE/2020/12830, Rev.2 guideline, as mean recoveries in the fortified samples were within the range 70-120%, measured against freshly prepared standards.

Boscalid residues were stable in honey extracts for at least 15 days of frozen storage.

Storage stability of matrix-matched standard solutions

A matrix-matched standard solution at 25 ng/mL was analysed after frozen storage, and the average response factor (5 injections) obtained was compared with the average response factor (5 injections) obtained for a freshly prepared solution.

The difference between average response factors from at least 5 replicate measurements for each of the two solutions did not differ by more than 10%.

Boscalid residues were stable in honey matrix matched calibration solutions for at least 14 days of frozen storage.

Characteristics for the analytical method used for validation of boscalid residues in honey

Selectivity and specificity	Representative, clearly labelled chromatograms of standard at the lowest calibrated level, untreated sample and sample fortified at the lowest fortification level for both primary and confirmatory methods were provided to prove selectivity of the method. Mass spectrum was provided to justify the selection of ions used for determination. Untreated samples (non-fortified samples) were determined from the matrix used in fortification experiments and the boscalid residues were not higher than 30% of the LOQ for both primary and confirmatory methods.
Calibration	Ranged from 0.77 ng/mL to 30.5 ng/mL (corresponding to 0.003 to 0.12 mg/kg). $R^2 = 0.99964$ for primary method (343.1 > 307.0) $R^2 = 0.99940$ for confirmatory method (343.1 > 271.0)
Assessment of matrix effects is presented	yes
Limit of determination (LOD)	0.01 mg/kg
Limit of quantification (LOQ)	0.77 ng/mL (corresponding to 0.003 mg/kg).

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method confirmed that this method is suitable for analysis the content of the active substance bos-

calid in honey.

A 1.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 1.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.
Please refer to point A.2.1.1.3.3

A 1.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.7 A.2.A.9 Other Studies/ Information

No new or additional studies have been submitted.