

# **REGISTRATION REPORT**

## **Part B**

### **Section 5**

#### **Analytical Methods**

Detailed summary of the risk assessment

Product code: GLOB1811F

Product name(s): RASPUT

Chemical active substance:

Boscalid, 500 g/kg

**Poland – Art. 33**

#### **CORE ASSESSMENT**

**(authorization)**

Applicant: Globachem NV

Submission date: June 2021

**MS Finalisation date: 18/03/2022**

## Version history

When	What
December 2021	First zRMS PL evaluation
March 2022	RR finalized by zRMS after commenting period

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## 5 Analytical methods

### Review Comments:

This application was submitted by Globachem NV for approval of Rasput (GLOB1811F) a water dispersible granule (WG) containing 500 g/kg boscalid for use as a fungicide in oilseed rape in Poland.

Boscalid was included on Annex I of Directive 91/414/EEC on 1 of August 2008 under Inclusion Directive 2008/44/EC.

This Part B document only reviews data (Annex III) and additional information that has not previously been considered within the EU review process.

Since this document is based on the information provided by the applicant, all review comments, additions and corrections have been made using commenting boxes or highlighted in grey. Any incorrect data or text not evaluated by the zRMS has been crossed out.

### 5.1 Conclusion and summary of assessment

Sufficient data on Analytical Methods are available for the plant protection product RASPUT 50% WG and the technical active substance: boscalid. The data presented in analytical methods cover the required information in compliance with the intended use of RASPUT 50% WG in selected countries of the central zone.

No data gaps are noticed.

This RR is evaluated in accordance with the Regulation (EC) No 284/2013.

Sufficiently sensitive and selective analytical methods are available for the active substance(s) and relevant impurities in the plant protection product.

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

Commodity/crop	Supported/ Not supported
Oilseed rape (Winter & Spring)	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: The method (XXX T., 2018, report no DNA4730, KCP 5.1.1-01) and its validation are acceptable for the determination of boscalid in the product RASPUT 50% WG according to DG Sanco 3030/99 rev. 4, July 2000. The LOQ recovery showed results between 96.05% to 102.2% with a mean of 98.69% and a standard deviation of 2.573.

This submitted study has been validated in a proper manner.

Reference:	KCP 5.1.1-01
Report	Validation of the method of determination of Boscalid in a water dispersible granule formulation, in compliance with good laboratory practice, XXX T., 2018, DNA4730, David Norris Analytical Laboratories Ltd..
Guideline(s):	SANCO/3030/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Materials and methods

The assay of Boscalid was performed using approximately 0.025 g of formulated material. The mass of the formulation was accurately recorded, transferred to a 50 mL volumetric flask. The sample was partially made up to volume with a 50:50 mixture of Acetonitrile and Deionized water and sonicated for 2 minutes. Once cooled back to room temperature the sample was made to volume with a 50:50 mixture of Acetonitrile and Deionized water. These solutions were then used for assay by injecting each solution once into the HPLC-DAD under the following conditions:

#### HPLC-DAD Conditions:

Instrument:	<del>Agilent 1100 Series HPLC-DAD</del> Agilent 1200 Series HPLC-DAD
Mode:	Isocratic Reverse Phase
Column:	Grace C18, 250 mm x 4.6 mm
Packing:	C18, 5 µm
Eluent:	55 % Acetonitrile 45 % Water adjusted to pH 3 with Phosphoric Acid
Wavelength:	280 nm
Flow Rate:	1.0 mL/min
Injection Volume:	10 µL
Column Temperature:	25 °C
Data Collection:	Chemstation

Retention Time: Approximately 11.7 to 11.8 minutes

LC-MS Q-ToF LC Conditions – MS Spectral Analysis:

Instrument: Agilent 1200 Series HPLC-DAD  
Mode: Isocratic Reverse Phase  
Column: Grace C18, 250 mm x 4.6 mm  
Packing: C18, 5 µm  
Eluent: 55 % Acetonitrile  
45 % Water adjusted to pH 3 with Phosphoric Acid  
Wavelength: 280 nm  
Flow Rate: 1.0 mL/min  
Injection Volume: 10 µL  
Column Temperature: 25 °C  
Data Collection: Mass Hunter  
Retention Time: Approximately 11.8 minutes

LC-MS Q-ToF LC Conditions – MS Spectral Analysis:

Instrument: Agilent 6500 Series Q-ToF Mass Spectrometer  
Mode: ESI Jet Spray Source  
Ionisation: Positive  
MS Scan Range: 50-1000 m/z  
MS/MS Scan range: 50-500 m/z  
Extracted Ions: N/A (Full Scan)  
Acquisition Rate: 1 Spectra/Second  
Acquisition Time: 1000 ms/Spectra

Gas Temperature: 250 °C  
Drying Gas Flow: 7 L/min  
Nebulizer: 40 psig  
Sheath Gas: 250 °C  
Sheath Gas Flow: 7 L/min  
Collision Energy: 0 to 30 V  
VCAP: 3000 V  
Nozzle Voltage: 2000 V  
Fragmenter: 100 V  
Skimmer: 65 V  
OCT 1 RF Vpp: 750 V

Data Acquisition: Mass Hunter

**Validation - Results and discussions**

**Table 5.2-1: Methods suitable for the determination of active substance Boscalid in plant protection product GLOB1811F**

	Boscalid
Author(s), year	XXX T., 2018
Principle of method	HPLC-DAD

	<b>Boscalid</b>
<b>Linearity</b> (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	Tested from eighteen injections of nine levels of standard ranging from a blank to 1.0 mg/mL. The method is linear with a correlation coefficient of 1.0000.
<b>Precision – Repeatability Mean</b> <b>n = 6</b> (%RSD)	The method is repeatable with a sample precision ranging from 502.3 g/kg to 507.2 g/kg, a mean of 504.2 g/kg, a standard deviation of 1.844 and a percentage relative standard deviation of 0.366 %.
<b>Accuracy</b> <b>n = 6</b> (% Recovery)	The method is accurate with values of percentage recovery ranging from 98.61 % to 100.8 %, a mean of 99.54 % and a standard deviation of 0.936.
<b>Interference/ Specificity</b>	<p>The method was found to be specific by comparing a Boscalid reference standard and a sample of GLOB1811F, both prepared in a 50:50 mixture Acetonitrile and Deionised water. Analysis was made by HPLC-DAD and LCMS Q-ToF.</p> <p><u>UV-Spectral Analysis</u> The Boscalid reference standard gave a peak at 11.7 minutes with a spectral maximum at 195 nm and a secondary maximum at 230 nm, reducing to extinction by 320 nm. The GLOB1811F sample also gave a peak at 11.7 minutes with a spectral maximum at 195 nm and a secondary maximum at 230 nm, reducing to extinction by 320 nm in a similar manner to the reference standard. The method is shown to be specific for Boscalid.</p> <p><u>MS Spectral Analysis</u> The Boscalid reference standard gave a peak at 11.8 minutes showing the molecular ion of <math>[M-H]^+</math> at 343 m/z, with fragment ions present at <math>[M-H]^+</math> at 140 m/z, 272 m/z and 307 m/z. The GLOB1811F sample also gave a peak at 11.8 minutes showing the molecular ion of <math>[M-H]^+</math> at 343 m/z, with fragment ions present at <math>[M-H]^+</math> at 140 m/z, 272 m/z and 307 m/z, in a similar manner to the reference standard. The method is shown to be specific for Boscalid.</p> <p>There were no analyte interferences.</p>
<b>Comment</b>	/

## Conclusion

The analytical method is suitable for the specific and accurate determination of Boscalid in GLOB1811F, with acceptable accuracy and precision. The validation complies with the criteria of SANCO/3030/99 rev. 4.

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

There are no relevant impurities which are of toxicological, ecotoxicological and/or environmental concern.

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

Under current EU legislation, methods on formulants are not required. However, if a formulant is defined as relevant for toxicity (environment, health), then a method needs to be provided. There are however no formulants in GLOB1811F that are defined as relevant for toxicity.

### **5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)**

A CIPAC method No. 673 is available for Boscalid.

### **5.2.2 Methods for the determination of residues (KCP 5.1.2)**

Please refer to post-registration methods.

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

### **5.3.1 Analysis of the plant protection product (KCP 5.2)**

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

### **5.3.2 Description of analytical methods for the determination of residues 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 not identical.

Indeed, the previous residue definition for Boscalid was different for all the commodities under the pesticide-code 1000000 (products of animal origin-terrestrial animals), where residue definition was sum of Boscalid and its hydroxy metabolite 2-chloro-N-(4'-chloro-5-hydroxybiphenyl-2-yl)nicotinamide (free and conjugated) expressed as Boscalid, and now the residue definition is different for pesticide-code number 1000000 except 1040000, 1011010, 1011020, 1011050, 1012010, 1012020, 1012050, 1013010, 1013020, 1013050, 1014010, 1014020, 1014050, 1015010, 1015020, 1015050, 1016010, 1016020, 1017010, 1017020, 1017050, 1020000 and 1030000.

**Table 5.3-1: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required**

<b>Matrix</b>	<b>Residue definition</b>	<b>MRL / limit</b>	<b>Reference for MRL/level Remarks</b>
Plant, high water content	Boscalid	0.01 mg/kg	Reg. (EU) 2016/156
Plant, high acid content		0.01 mg/kg	Reg. (EU) 2016/156
Plant, high protein/high starch content (dry commodities)		3.0 mg/kg	Reg. (EU) 2016/156



Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high oil content		0.01 mg/kg	Reg. (EU) 2016/156
Plant, difficult matrices (hops, spices, tea)		0.01 mg/kg	Reg. (EU) 2016/156
Muscle	Sum of Boscalid and its hydroxy metabolite 2-chloro-N-(4'-chloro-5-hydroxybiphenyl-2-yl)nicotinamide (free and conjugated) expressed as Boscalid (except for the pesticide-code numbers mentioned above)	0.01 mg/kg	Reg. (EU) 2016/156
Milk		0.02 mg/kg	Reg. (EU) 2016/156
Eggs		0.01 mg/kg	Reg. (EU) 2016/156
Fat		0.07 mg/kg	Reg. (EU) 2016/156
Liver, kidney		0.05 mg/kg	Reg. (EU) 2016/156
Soil (Ecotoxicology)	Boscalid	0.05 mg/kg	Common limit
Drinking water (Human toxicology)	Boscalid	0.1 µg/L	General limit for drinking water
Surface water (Ecotoxicology)	Boscalid	125 µg/L	Lowest NOEC from aquatic toxicity study on <i>O. mykiss</i>
Air	Boscalid	30 µg/m <sup>3</sup>	AOEL sys/AOEL inhal: 0.1 mg/kg bw/d
Tissue (meat or liver)	-	Not required	Not classified as T / T+
Body fluids		Not required	Not classified as T / T+

### 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.

**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 0.05 mg/kg	GC-MS  HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2000)
	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, 2000)

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 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, 2000)
	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, 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 0.05 mg/kg	GC-MS  HPLC-MS/MS	DAR 2002 (Weeren and Pelz, 1999) DAR 2002; FAO, 2006 (Funk and Mackenroth, 2000)
	ILV	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)
	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, 2000)
Difficult (if required, depends on intended use)	Primary	0.01 mg/kg	GC-MS	DAR 2002 (Reichert, 2001)

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

**Table 5.3-3: Statement on extraction efficiency**

	Method for products of plant origin
Required, available from:	Mackenroth-Lehmann, 2007a
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.

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

<b>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</b>				
<b>Matrix type</b>	<b>Method type</b>	<b>Method LOQ</b>	<b>Principle of method (i.e. GC-MS or HPLC-UV)</b>	<b>Author(s), year / missing</b>
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)
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)

**Table 5.3-5: Statement on extraction efficiency**

	<b>Method for products of animal origin</b>
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.

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)

### 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.

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	GC-MS	DAR 2002 (Keller, 1998b)
Surface water	Primary	0.5 mg/kg	GC-MS	DAR 2002 (Grote, 2001)

### 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.

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 <sup>3</sup>	GC-MS	DAR 2002 (Zangmeister, 2000)

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

Boscalid is not classified as toxic or highly toxic, no residue method for body fluids and tissues is required.

### 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

### List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1-01	XXX, T.	2018	Validation of the method of determination of Boscalid in a water dispersible granule formulation, in compliance with good laboratory practices. DNA4730 David Norris Analytical Laboratories Ltd. GLP Unpublished	N	Globachem NV
KCP 5.2-01	XXX, D.	2021a	Acute toxicity of GLOB1811F to <i>Daphnia magna</i> in a 48-hour static test: Verification of the concentration of Boscalid in the test solutions. 20 35 CRA 0080 BioChem agrar, Labor für biologische und chemische Analytik GmbH GLP Unpublished	N	Globachem NV
KCP 5.2-02	XXX, D.	2021b	Effects of GLOB1811F on <i>Lemna gibba</i> in a growth inhibition test under semi-static test conditions: Verification of the concentration of Boscalid in the test solutions. 20 35 CRA 0078 BioChem agrar, Labor für biologische und chemische Analytik GmbH GLP Unpublished	N	Globachem NV
KCP 5.2-03	XXX, D.	2021c	Effects of GLOB1811F on <i>Pseudokirchneriella subcapitata</i> in an algal growth inhibition test: Verification of the concentration of Boscalid in the test solutions. 20 35 CRA 0079 BioChem agrar, Labor für biologische und chemische Analytik GmbH GLP Unpublished	N	Globachem NV
KCP 5.2-04	XXX, M.	2021	GLOB1811F – Repeated exposure of honey bee ( <i>Apis mellifera</i> L.) larvae under laboratory	N	Globachem NV

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title</b> <b>Company Report No.</b> <b>Source (where different from company)</b> <b>GLP or GEP status</b> <b>Published or not</b>	<b>Vertebrate study</b> <b>Y/N</b>	<b>Owner</b>
			conditions: Determination of boscalid concentrations in final diets. 20 35 CRB 0161 BioChem agrar, Labor für biologische und chemische Analytik GmbH GLP Unpublished		

## **Appendix 2 Detailed evaluation of submitted analytical methods**

### **A 2.1 Analytical methods for Boscalid**

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

No new or additional studies have been submitted.

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

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

#### A 2.1.2.7 Other Studies/ Information

Comments of zRMS: The analytical phase of the study XXX D., 2021a, report No 20 35 CRA 0080, Acute toxicity of GLOB1811F to *Daphnia magna* in a 48-hour static test and it's validation are acceptable for verification of the concentrations of the active ingredient Boscalid in the test solutions. The study has been performed in compliance with Good Laboratory Practice and SANTE/2020/12830, Rev.1.

Reference:	KCP 5.2-01 (Study submitted as KCP 10.2.1-01)
Report	Acute toxicity of GLOB1811F to <i>Daphnia magna</i> in a 48-hour static test: Verification of the concentration of Boscalid in the test solutions, XXX D., 2021a, report No 20 35 CRA 0080.
Guideline(s):	SANTE/2020/12830, Rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

#### Executive summary

The purpose of the analytical phase of the study was the verification of the concentrations of the active ingredient Boscalid of the test item GLOB1811F, in an acute toxicity test of *Daphnia magna* (BioChem Project No. 20 48 ADL 0023).

A reversed phase HPLC method with MS/MS-detection for the determination of the active ingredient in the aquatic test matrix was validated according to the guidance document SANTE/2020/12830, Rev.1 and used for the analytical determination.



## Materials and methods

### HPLC conditions:

Instrument	A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used
Mobile phase	A: 0.1% formic acid and 5 mmol ammonium formate in water B: 0.1% formic acid and 5 mmol ammonium formate in methanol
Flow rate	0.25 mL/min
Gradient	0.00 min 70% B 60% B 4.00 min 85% B 4.50 min 100% B 5.50 min 100% B 5.51 min 60% B 8.50 min 60% B
Run time	8.50 min
Injection Volume	5 µL
Column	ACE Excel3 C18-AR, 3 µm, 100 * 2.1 mm
Detection	Boscalid: ESI positive, [M+H] <sup>+</sup> ; MRM: m/z 343.0->306.95 (Quantifier); 343.0->270.95; 343.0->271.95
Retention Time:	5.1-5.2 min

## Results and discussion

All reported concentrations are pure Boscalid, i.e. they were corrected for purity.

An external matrix-matched calibration with the analytical reference item including 8 calibration levels was performed.

### *Calibration data Boscalid*

The detector signal for Boscalid (mass transition 343.0->306.95) was linear in the range from 1.131 to 37.69 µg/L. The corresponding calibration range regarding overall dilution ( $DF_{\text{analytical}} = 200$  for lower calibration limit and  $DF_{\text{analytical}} = 1053$  for upper calibration limit) was from 226.1 to 39671 µg/L. The equation of the calibration curve was  $Y = 190868186661X + 16336$ . The correlation factor  $r^2$  was 0.99991.

### *Method validation data*

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level in test matrix. The limit of detection (LOD), defined in the context of this phase of the study as the lowest successfully calibration level in diluent, was 1.131 µg/L for Boscalid.

### *Method validation results Boscalid*

Validation level	Nominal concentration [µg/L]	Analysed concentration [µg/L]	Mean analysed concentration [µg/L]	Mean recovery [% of nominal concentration]	RSD [%]
Blank	0.000	nd < LOD	-	-	-
Low (LOQ)	766.8	745.7 754.7 750.7	747.0	97.4	1.1

		750.9			
		733.1			
		32736			
		31416			
High	32629	32091	32170	98.6	1.5
		32258			
		32349			

nd – Boscalid was not detected; LOQ = 766.8 µg/L; LOD = 1.131 µg/L

Concentrations of the target analytes in the blanks were < of its respective method LOD (i.e. 1.131 µg/L for Boscalid).

The mean recovery values are 97.4 and 98.6%. The corresponding relative standard deviation (RSD) values were below 20%.

#### Analysis results of Boscalid in test samples

Treatment	Nominal concentration [µg/L]	Dilution factor	Analysed concentration [µg/L]	Recovery [% of nominal]	Analysed concentration [µg/L]	Recovery [% of nominal]
			0 h fresh		48 h spent	
1	0.000	400	< LOD	-	< LOD	-
2	3109	400	3051	98.1	2827	90.9
3	6219	400	5834	93.8	5353	86.1
4	12438	533	12036	96.8	10984	88.3
5	24876	1067	23439	94.2	24196	97.3
6	49699	2105	49995	100.6	53370	107.4

LOQ = 766.8 µg/L; LOD = 1.131 µg/L

Recoveries from fresh test samples were in the range from 93.8 to 100.6%. The nominal concentrations at test start were therefore confirmed. Recoveries from the spent samples were in the range from 88.3 to 107.4%.

#### Characteristics for the analytical method used for validation of ~~Difenoconazole~~ Boscalid residues in OECD medium

	<b><del>Difenoconazole</del> Boscalid</b>
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 70% of the lowest to 120% of the highest validation concentration. N = 8
Calibration range	Calibration range: 1.131 to 37.69 µg/L R <sup>2</sup> > 0.99991
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/2020/12830.
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. <b>LOQ = 1.131 µg/L</b> <b>LOD = 1.131 µg/L</b>

*Characteristics for the analytical method used for validation of ~~Difenoconazole~~ Boscalid residues in OECD medium*

	<b>Difenoconazole-Boscalid</b>
	LOQ = 766.8 µg/L

### Conclusion

The specificity of the method was assured by MS/MS-detection and the absence of interfering peaks. The recovery and precision data show that the method meets the requirements of the guidance document SANTE/2020/12830, Rev.1; all criteria are fulfilled:

- control values do not exceed 30% of the method LOQ,
- mean recoveries at each level are in the range 70-120%,
- the RSD is < 20% per level.

Comments of zRMS: The analytical phase of the study XXX D., 2021b, report No 20 35 CRA 0078, Effects of GLOB1811F (RASPUT 50% WG) on *Lemna gibba* in a growth inhibition test under semi-static test conditions and it's validation are acceptable for verification of the concentrations of the active ingredient Boscalid in the test solutions.

The study has been performed in compliance with Good Laboratory Practice and SANTE/2020/12830, Rev.1.

Reference:	KCP 5.2-02 (Study submitted as KCP 10.2.1-02)
Report	Effects of GLOB1811F on <i>Lemna gibba</i> in a growth inhibition test under semi-static test conditions: Verification of the concentration of Boscalid in the test solutions, XXX D., 2021b, report No 20 35 CRA 0078.
Guideline(s):	SANTE/2020/12830, Rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Executive summary

The purpose of the analytical phase of the study was the verification of the concentrations of the active ingredient Boscalid of the test item GLOB1811F, in an acute toxicity test of *Lemna gibba* (BioChem Project No. 20 48 ALE 0020).

A reversed phase HPLC method with MS/MS-detection for the determination of the active ingredient in the aquatic test matrix was validated according to the guidance document SANTE/2020/12830, Rev.1 and used for the analytical determination.

### Materials and methods

### HPLC conditions:

Instrument	A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used
Mobile phase	A: 0.1% formic acid and 5 mmol ammonium formate in water B: 0.1% formic acid and 5 mmol ammonium formate in methanol
Flow rate	0.25 mL/min
Gradient	0.00 min 70% B 60% B 4.00 min 85% B 4.50 min 100% B 5.50 min 100% B 5.51 min 60% B 8.50 min 60% B
Run time	8.50 min
Injection Volume	5 µL
Column	ACE Excel3 C18-AR, 3 µm, 100 * 2.1 mm
Detection	Boscalid: ESI positive, [M+H] <sup>+</sup> ; MRM: m/z 343.0->306.95 (Quantifier); 343.0->270.95; 343.0->271.95
Retention Time:	5.1-5.3 min

### Results and discussion

All reported concentrations are pure Boscalid, i.e. they were corrected for purity.

An external matrix-matched calibration with the analytical reference item including 8 calibration levels was performed.

#### *Calibration data Boscalid*

The detector signal for Boscalid (mass transition 343.0->306.95) was linear in the range from 0.5125 to 30.15 µg/L. The corresponding calibration range regarding overall dilution ( $DF_{\text{analytical}} = 20$  for lower calibration limit and  $DF_{\text{analytical}} = 4000$  upper calibration limit) was from 10.25 to 120598 µg/L. The equation of the calibration curve was  $Y = 138741X + 266$ . The correlation factor  $r^2$  was 0.99998.

#### *Method validation data*

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level in test matrix. The limit of detection (LOD), defined in the context of this phase of the study as the lowest successfully calibration level in diluent, was 0.5125 µg/L for Boscalid.

#### *Method validation results Boscalid*

Validation level	Nominal concentration [µg/L]	Analysed concentration [µg/L]	Mean analysed concentration [µg/L]	Mean recovery [% of nominal concentration]	RSD [%]
Blank	0.000	nd nd	-	-	-
Low (LOQ)	34.71	32.82 34.65 34.07 33.72 34.13	33.88	97.6	2.0
High	97768	96427	93328	95.5	3.1

90743  
93423  
95870  
90175

nd – Boscalid was not detected; LOQ = 34.71 µg/L; LOD = 0.5125 µg/L

Concentrations of the target analytes in the blanks were < of its respective method LOD (i.e. 0.5125 µg/L for Boscalid).

The mean recovery values are 95.5 and 97.6%. The corresponding relative standard deviation (RSD) values were below 20%.

*Analysis results of Boscalid in test samples*

Treatment	Nominal concentration [µg/L]	Dilution factor	Analysed concentration [µg/L]	Recovery [% of nominal]	Analysed concentration [µg/L]	Recovery [% of nominal]
			0 h fresh		3 days spent	
1	40	0.0000	< LOD	-	nd	-
2	40	140.2	129.2	92.2	123.2	87.9
3	123	444.5	419.5	94.4	392.1	88.2
4	160	1423	1436	100.9	1315	92.4
5	500	4552	4693	103.1	2183	48.0
6	1600	14567	13262	91.0	5449	37.4
7	2500	46613	45953	98.6	17648	37.9
8	8000	149160	120059	80.5	45549	30.5
			3 days fresh		5 days spent	
1	40	0.0000	< LOD	-	nd	-
2	40	140.2	129.9	92.6	125.0	89.1
3	123	444.5	417.4	93.9	410.2	92.3
4	160	1423	1450	101.9	1257	88.4
5	500	4552	4689	103.0	2434	53.5
6	1600	14567	13274	91.1	7467	51.3
7	2500	46613	34325	73.6	14827	31.8
8	8000	149160	140981	94.5	55815	37.4
			5 days fresh		7 days spent	
1	40	0.0000	< LOD	-	nd	-
2	40	140.2	139.1	99.2	138.7	98.9
3	123	444.5	417.3	93.9	418.7	94.2
4	160	1423	1403	98.6	1284	90.2
5	500	4552	4563	100.2	2557	56.2
6	1600	14567	13743	94.3	6698	46.0
7	2500	46613	41680	89.4	20647	44.3
8	8000	149160	154182	103.4	69675	46.7

nd – Boscalid was not detected; LOQ = 34.71 µg/L; LOD = 0.5125 µg/L

Recoveries from fresh test samples were in the range from 73.6 to 103.4%. Recoveries from the spent samples were in the range from 30.5 to 98.9%.

*Characteristics for the analytical method used for validation of ~~Difenoconazole~~ Boscalid residues in OECD medium*

	<b>Difenoconazole Boscalid</b>
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 70% of the lowest to 120% of the highest validation concentration. N = 8
Calibration range	Calibration range: 0.5125 to 30.15 µg/L R <sup>2</sup> > 0.99998
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/2020/12830.
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. LOQ = 0.5125 µg/L LOD = 0.5125 µg/L LOQ = 34.71 µg/L

### Conclusion

The specificity of the method was assured by MS/MS-detection and the absence of interfering peaks. The recovery and precision data show that the method meets the requirements of the guidance document SANTE/2020/12830, Rev.1; all criteria are fulfilled:

- control values do not exceed 30% of the method LOQ,
- mean recoveries at each level are in the range 70-120%,
- the RSD is < 20% per level.

Comments of zRMS: The analytical phase of the study XXX D., 2021c, report No 20 35 CRA 0079, Effects of GLOB1811F (RASPUT 50% WG) on *Pseudokirchneriella subcapitata* in an algal growth inhibition test and its validation are acceptable for verification of the concentrations of the active ingredient Boscalid in the test solution.

The study has been performed in compliance with Good Laboratory Practice and SANCO/3029/99 rev. 4.

Reference:	KCP 5.2-03 (Study submitted as KCP 10.2.1-03)
Report	Effects of GLOB1811F on <i>Pseudokirchneriella subcapitata</i> in an algal growth inhibition test: Verification of the concentration of Boscalid in the test solutions, XXX D., 2021c, report No 20 35 CRA 0079.
Guideline(s):	SANCO/3029/99 rev. 4.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Executive summary

The purpose of the analytical phase of the study was the verification of the concentrations of the active ingredient Boscalid of the test item GLOB1811F, in an acute toxicity test of *Pseudokirchneriella subcapitata* (BioChem Project No. 20 48 AAL 0027).

A reversed phase HPLC method with MS/MS-detection for the determination of the active ingredient in the aquatic test matrix was validated according to the guidance document SANTE/2020/12830, Rev.1 and used for the analytical determination.

## Materials and methods

### HPLC conditions:

Instrument	A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used
Mobile phase	A: 0.1% formic acid and 5 mmol ammonium formate in water B: 0.1% formic acid and 5 mmol ammonium formate in methanol
Flow rate	0.25 mL/min
Gradient	0.00 min 70% B-60% B 4.00 min 85% B 4.50 min 100% B 5.50 min 100% B 5.51 min 60% B 8.50 min 60% B
Run time	8.50 min
Injection Volume	5 µL
Column	ACE Excel3 C18-AR, 3 µm, 100 * 2.1 mm
Detection	Boscalid: ESI positive, [M+H] <sup>+</sup> ; MRM: m/z 343.0->306.95 (Quantifier); 343.0->270.95; 343.0->271.95
Retention Time:	5.1-5.2 min

## Results and discussion

All reported concentrations are pure Boscalid, i.e. they were corrected for purity.

An external matrix-matched calibration with the analytical reference item including 8 calibration levels was performed.

### *Calibration data Boscalid*

The detector signal for Boscalid (mass transition 343.0->306.95) was linear in the range from 0.7350 to 31.96 µg/L. The corresponding calibration range regarding overall dilution ( $DF_{\text{analytical}} = 100$  for lower calibration limit and upper calibration limit) was from 73.50 to 3196 µg/L. The equation of the calibration curve was  $Y = 160342X + 54750$ . The correlation factor  $r^2$  was 0.99982.

### *Method validation data*

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully validated fortification level in test matrix. The limit of detection (LOD), defined in the context of this phase of the study as the lowest successfully calibration level in diluent, was 0.7350 µg/L for Boscalid.

### *Method validation results Boscalid*

Validation level	Nominal concentration [µg/L]	Analysed concentration [µg/L]	Mean analysed concentration [µg/L]	Mean recovery [% of nominal concentration]	RSD [%]
Blank	0.000	< LOD	-	-	-

< LOD					
Low (LOQ)	248.9	242.6	244.3	98.2	2.2
		248.1			
		251.8			
		238.3			
		241.0			
High	2620	2531	2451	93.5	2.1
		2411			
		2407			
		2434			
		2470			

LOQ = 248.9 µg/L; LOD = 0.7350 µg/L

Concentrations of the target analytes in the blanks were < of its respective method LOD (i.e. 248.9 µg/L for Boscalid).

The mean recovery values are 93.5 and 98.2%. The corresponding relative standard deviation (RSD) values were below 20%.

#### Analysis results of Boscalid in test samples

Treatment	Nominal concentration [µg/L]	Dilution factor	Analysed concentration [µg/L]	Recovery [% of nominal]	Analysed concentration [µg/L]	Recovery [% of nominal]
			0 h fresh		72 h spent	
1	200	0.0000	< LOD	-	< LOD	-
2	200	1038	967.9	93.2	973.8	93.8
3	133	1449	1304	90.0	1342	92.6
4	182	2029	1851	81.7	1824	80.5
5	250	2841	2616	92.1	2247	79.1
6	200	3978	3743	94.1	2666	67.0

LOQ = 248.9 µg/L; LOD = 0.7350 µg/L

Recoveries from fresh test samples were in the range from 81.7 to 94.1%. The nominal concentrations at test start were therefore confirmed. Recoveries from the spent samples were in the range from 67.0 to 93.8%.

#### Characteristics for the analytical method used for validation of ~~Difenoconazole~~ Boscalid residues in OECD medium

	<b>Difenoconazole Boscalid</b>
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 70% of the lowest to 120% of the highest validation concentration. N = 8
Calibration range	Calibration range: 0.7350 to 31.96 µg/L R <sup>2</sup> > 0.99982
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/2020/12830.



Characteristics for the analytical method used for validation of ~~Difenoconazole~~ Boscalid residues in OECD medium

	Difenoconazole Boscalid
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. LOQ = 0.7350 µg/L LOD = 0.7350 µg/L LOQ = 248.9 µg/L

## Conclusion

The specificity of the method was assured by MS/MS-detection and the absence of interfering peaks. The recovery and precision data show that the method meets the requirements of the guidance document SANTE/2020/12830, Rev.1; all criteria are fulfilled:

- control values do not exceed 30% of the method LOQ,
- mean recoveries at each level are in the range 70-120%,
- the RSD is < 20% per level.

Comments of zRMS: The analytical phase of the study Dreßler, K., 2021, report No 20 35 CRB 0162, Effects of GLOB1811F (RASPUT 50% WG) on Chronic toxicity of GLOB1811F to the honey bee *Apis mellifera* L. under laboratory conditions and it's validation are acceptable for verification of the concentrations of the active ingredient Boscalid in in feeding solutions.

The study has been performed in compliance with Good Laboratory Practice and SANCO/3029/99 rev. 4.

Reference:	KCP 5.2-04 (Study submitted as KCP 10.3.1.2)
Report	Chronic toxicity of GLOB1811F to the honey bee <i>Apis mellifera</i> L. under laboratory conditions: Determination of boscalid concentrations in feeding solutions, Dreßler, K., 2021, report No 20 35 CRB 0162.
Guideline(s):	<del>SANCO/3029/99 rev. 4 (11/07/2000)</del> SANCO/3029/99 rev. 4.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Executive summary

The purpose of the analytical phase of the study was the verification of the concentration of the active ingredient Boscalid in feeding solutions of a chronic toxicity test on honey bees. The determination was conducted by an in-house developed method using reversed phase high performance liquid chromatography (RP-HPLC) with DAD-detection (diode array detector).

The analytical method was validated according to SANCO/3029/99 rev. 4.

## Materials and methods

A Shimadzu LC-20 HPLC system equipped with a diode-array detector was used:

LC-Instrument	Manufacturer	Model
Degasser	Shimadzu	DGU-20A5R
Pumps	Shimadzu	LC-20ADXR

Auto sampler	Shimadzu	SIL-20ACXR
Column oven	Shimadzu	CTO-20AC
Diode-array detector	Shimadzu	SPD-M20A
Communications bus module	Shimadzu	CBM-20A
Data System	Shimadzu	LABSOLUTIONS WS - SINGLE PDA (VERS

The following HPLC parameters were used for analysis of the samples:

Parameter	Characteristics
Mobile phase	A: Water with 0.1% H <sub>3</sub> PO <sub>4</sub> B: Acetonitrile with 0.1% H <sub>3</sub> PO <sub>4</sub>
Flow rate	0.4 mL/min
Gradient	0.00 min 15% B 6.00 min 90% B 8.00 min 90% B 8.01 min 15% B 10.00 min stop
Column	Phenomenex Kinetex EVO C18 2.1mm x 100, 2.6µm
Detection	The signal was recorded at 225nm.
Retention time	Approx. 4.7 min for Boscalid

#### Preparation of solutions

Solution	Preparation																																		
HPLC eluent A	1 mL phosphoric acid was added to 1 L ultrapure water																																		
HPLC eluent B	1 mL phosphoric acid was added to 1 L acetonitrile																																		
Sample matrix	50% (w/v) sucrose solution containing 0.1% (w/v) xanthan																																		
Dilution medium	100% blank extract (Extract of 0.2 g untreated sample matrix)																																		
REF-2020/0418 stock	11.87 mg of Boscalid (99.8% according to certificate of analysis) were weighed into a 10 mL measuring flask and filled to the mark with methanol (concentration of Boscalid = 1184.6 mg/L).																																		
REF-2020/0418 stock -Dil 1	0.160 mL of REF-2020/0418 stock was pipetted into a 10 mL flask and filled to the mark with Blank extract (concentration of Boscalid = 18.95 mg/L).																																		
Calibration solutions	<div>The following volumes were mixed in autosampler vials:</div> <table><tr><th>Calibration solutions</th><th>Volume [mL]</th><th>Original solution</th><th colspan="2">Diluted</th><th>Conc. of Boscalid [mg/L]</th></tr><tr><td></td><td></td><td></td><th>with [mL]</th><th>with</th><td></td></tr><tr><td>2035CRB0162-Cal 1</td><td>0.200</td><td rowspan="5">REF-2020/0418 stock -Dil 1</td><td>0.800</td><td rowspan="5">Blank extract</td><td>3.79</td></tr><tr><td>2035CRB0162-Cal 2</td><td>0.400</td><td>0.600</td><td>7.58</td></tr><tr><td>2035CRB0162-Cal 3</td><td>0.600</td><td>0.400</td><td>11.37</td></tr><tr><td>2035CRB0162-Cal 4</td><td>0.800</td><td>0.200</td><td>15.16</td></tr><tr><td>2035CRB0162-Cal 5</td><td>1.000</td><td>0.000</td><td>18.95</td></tr></table>	Calibration solutions	Volume [mL]	Original solution	Diluted		Conc. of Boscalid [mg/L]				with [mL]	with		2035CRB0162-Cal 1	0.200	REF-2020/0418 stock -Dil 1	0.800	Blank extract	3.79	2035CRB0162-Cal 2	0.400	0.600	7.58	2035CRB0162-Cal 3	0.600	0.400	11.37	2035CRB0162-Cal 4	0.800	0.200	15.16	2035CRB0162-Cal 5	1.000	0.000	18.95
Calibration solutions	Volume [mL]	Original solution	Diluted		Conc. of Boscalid [mg/L]																														
			with [mL]	with																															
2035CRB0162-Cal 1	0.200	REF-2020/0418 stock -Dil 1	0.800	Blank extract	3.79																														
2035CRB0162-Cal 2	0.400		0.600		7.58																														
2035CRB0162-Cal 3	0.600		0.400		11.37																														
2035CRB0162-Cal 4	0.800		0.200		15.16																														
2035CRB0162-Cal 5	1.000		0.000		18.95																														
Validation solutions	<div>Validation solutions</div> <div>20 35 CRB 0162-Val-stock</div> <div>The test item GLOB1811F was used for validation of the method.</div> <div>145.50 mg of GLOB1811F (49.72% Boscalid calculated considering the concentration of 497.2 g/L given in</div>																																		

2035 CRB 0162-Val-Dil1	the certificate of analysis) were weighed into a 10 mL measuring flask and filled to the mark with sample matrix (concentration of Boscalid = 7234.3 mg/L).
2035 CRB 0162-Val High	0.260 mL of 20 35 CRB 0162-Val-stock were pipetted into a 10 mL flask and filled to the mark with sample matrix (concentration Boscalid: 188.09 mg/L)
2035 CRB 0162-Val Low	Solution 20 35 CRB 0162-Val-stock was used as high validation level (concentration of Boscalid: 6079.2 mg/kg)
	Solution 20 35 CRB 0162-Val-Dil1 was used as low validation level (concentration of Boscalid: 158.1 mg/kg)

## Results and discussion

### Validation summary

Sample description	Number of replicates	Nominal conc. of Boscalid [mg/kg]	Mean analysed conc. of Boscalid [mg/kg]	Mean recovery [% of nominal]	RSD [%]
Low concentration (LOQ)	5	158	157	99.2	1.44
High concentration	5	6079	5729	94.2	1.82
Blank concentration	2	0.000	< 30% LOQ	-	-

LOQ = 158 mg/kg of Boscalid

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

### Limit of Quantification

The limit of quantification (LOQ) was defined in the context of this phase of the study as the lowest successfully verified fortification level of 158 mg/kg Boscalid in sample matrix sucrose solution containing 50% (w/v) sucrose with 0.1% xanthan (w/v) corresponding to 6.12 mg/L in diluted extracts.

## Analysis results

### Analysis results for Boscalid

Sample Name	Nominal* conc. of a.i. [mg/kg]	Analysed conc. of a.i. [mg/kg]	REC [%]
20BAC0096-D0-BC-A	0.000	< 30% LOQ	-
20BAC0096-D1-BC-A		< 30% LOQ	-
20BAC0096-D2-BC-A		< 30% LOQ	-
20BAC0096-D3-BC-A		< 30% LOQ	-
20BAC0096-D4-BC-A		< 30% LOQ	-
20BAC0096-D5-BC-A		< 30% LOQ	-
20BAC0096-D6-BC-A		< 30% LOQ	-
20BAC0096-D7-BC-A		< 30% LOQ	-
20BAC0096-D8-BC-A		< 30% LOQ	-
20BAC0096-D9-BC-A		< 30% LOQ	-

20BAC0096-D0-ET-A	317	308	97.1
20BAC0096-D1-ET-A		298	94.1
20BAC0096-D2-ET-A		316	100
20BAC0096-D3-ET-A		305	96.4
20BAC0096-D4-ET-A		316	100
20BAC0096-D5-ET-A		317	100
20BAC0096-D6-ET-A		314	99.2
20BAC0096-D7-ET-A		306	96.8
20BAC0096-D8-ET-A		317	100
20BAC0096-D9-ET-A		319	101
20BAC0096-D0-AT-A	5064	4877	96.3
20BAC0096-D1-AT-A		4725	93.3
20BAC0096-D2-AT-A		4905	96.9
20BAC0096-D3-AT-A		4770	94.2
20BAC0096-D4-AT-A		4927	97.3
20BAC0096-D5-AT-A		4783	94.5
20BAC0096-D6-AT-A		4788	94.6
20BAC0096-D7-AT-A		4820	95.2
20BAC0096-D8-AT-A		4862	96.0
20BAC0096-D9-AT-A		4803	94.9

LOQ = 158 mg/kg of Boscalid

\*Nominal conc. is based on analysed content of a.i. according to certificate of analysis.

The recoveries for Boscalid were between 93.3% and 101% in the feeding solutions. In the control specimens, the concentrations of the active ingredient were below 30% of LOQ.

## Conclusion

The recovery and precision data show that the influences of sample matrix were within the limits of the guidance document SANCO/3029/99 rev.4; all criteria were fulfilled:

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

Comments of zRMS: The analytical phase of the study XXX, M., 2021, report No 20 35 CRB 0161, Repeated exposure of honey bee (*Apis mellifera* L.) larvae under laboratory conditions Determination of boscalid concentrations in final diets and it's validation are acceptable for verification of the concentrations of the active ingredient Boscalid in final diets.

The study has been performed in compliance with Good Laboratory Practice and SANTE/2020/12830, Rev.1.

Reference:	KCP 5.2-05 (Study submitted as KCP 10.3.1.3)
Report	GLOB1811F – Repeated exposure of honey bee ( <i>Apis mellifera</i> L.) larvae under laboratory conditions Determination of boscalid concentrations in final diets, XXX, M., 2021, report No 20 35 CRB 0161.
Guideline(s):	SANTE/2020/12830, Rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Executive summary

The purpose of the analytical phase of the study was the verification of the concentration of boscalid in final diets (50/50 w/w royal jelly/ASS) of a honey bee larvae chronic toxicity test. The determination was conducted by an in-house developed method using reversed phase high performance liquid chromatography (RP-HPLC) coupled to a tandem mass spectrometer (LC-MS/MS).

The specimens contained two different aqueous sugar solutions (ASS):

ASS for diet B: 15% (w/v) glucose, 15% (w/v) fructose and 3% (w/v) yeast extract

ASS for diet C: 18% (w/v) glucose, 18% (w/v) fructose and 4% (w/v) yeast extract

The analytical method was validated according to SANTE/2020/12830, Rev. 1.

## Materials and methods

### Chromatographic system and mass spectrometer

An Agilent 1200 HPLC system with a 6460 triple quadrupole mass spectrometric detector was used for determination.

LC-Instrument	Manufacturer	Model
Binary pump	Agilent	G1312B
Degaser	Agilent	G1379B
Autosampler	Agilent	G1367C
Column oven	Agilent	G1316B
Diode array detector	Agilent	G1315C
MS detector	Agilent	G6460B
ESI Ion source	Agilent	G1948B
Data System	Agilent MassHunter	Data Acquisition for Triple Quad Version B.06.00 Quantitative Analysis for QQQ Version B.6.00

The following HPLC parameters were used for analysis of the samples:

Parameter	Characteristics
Mobile phase	A: Water with 0.1% formic acid + 5 mM ammonium formate B: Methanol with 0.1% formic acid
Flow rate	0.30 mL/min
Gradient	0.00 min 30% B 5.00 min 100% B 6.00 min 100% 6.01 min 30% 8.00 min stop
Column	Zorbax Eclipse C18; 2.1 µm x 50 mm
Detection	ESI positive, MRM m/z 343 → 307 m/z 343 → 271
Retention time	Approx. 5.2 min

### Preparation of solutions

Solution	Preparation
HPLC eluent A	1 mL formic acid and 5 mM ammonium formate were added to 1 L ultrapure water
HPLC eluent B	1 mL formic acid was added to 1 L methanol

Sample matrix	D3: ASS for diet B: 15% (w/v) glucose, 15% (w/v) fructose and 3% (w/v) yeast extract D4-D6: ASS for diet C: 18% (w/v) glucose, 18% (w/v) fructose and 4% (w/v) yeast, mixed with royal jelly 50/50 (w/w)					
Blank extract	Extract of untreated ASS for diet C					
Dilution medium	10/40/50 (v/v/v) blank extract/acetonitrile/water					
Stock solution 2020/0418-1	10.07 mg of boscalid (99.8% according to certificate of analysis) were weighed into a 10 mL measuring flask and filled to the mark with MeOH (concentration of boscalid = 1005 mg/L).					
REF 20CRB0161-Dil 1	0.050 mL of 2020/0418-1 were pipetted into a 5 mL flask and filled to the mark with methanol (concentration of boscalid = 10050 µg/L).					
REF 20CRB0161-Dil 2	0.050 mL of REF 20CRB0161-Dil 1 were pipetted into a 10 mL flask and filled to the mark with dilution medium (concentration of boscalid = 50 µg/L).					
Calibration solutions	The following volumes were mixed in autosampler vials:					
	Calibration solutions	Volume [mL]	Original solution	Diluted		Conc. of boscalid [µg/L]
				with [mL]	with	
	20CRB0161-Cal-1	0.035	REF 20CRB0161-Dil 2	0.965	Dilution medium	1.76
	20CRB0161-Cal-2	0.060		0.940		3.01
	20CRB0161-Cal-3	0.200		0.800		10.0
	20CRB0161-Cal-4	0.500		0.500		25.1
	20CRB0161-Cal-5	0.700		0.300		35.2
	20CRB0161-Cal-6	1.000		0.000		50.2
Validation solutions	The test item and the most complex sample matrix (ASS for diet C mixed with royal jelly 50/50 (w/w)) were used for validation of the method.					
	2020/0390-stock: 38.0 mg of GLOB1811F (49.72% boscalid as described in the certificate of analysis) were weighed into a 25 mL measuring flask and filled to the mark with ASS for diet C (concentration of boscalid = 756 mg/L).					
	Validation dilutions					
	Solution	Volume [mL]	Original solution	to [mL]	Diluted with	Conc. of boscalid [mg/L]
	20CRB0161-Val-Dil1	0.500	20CRB0161-Val-stock	5	ASS for diet C	76
	20CRB0161-Val-Dil2	0.235	20CRB0161-Val-Dil1	5		3.55
	2020/0390-stock and 20CRB0161-Val-Dil2 were mixed in a 1:1 (m/m) ratio with royal jelly to obtain the validation samples.					
	Validation samples:					
	Solution	Replicates	Original solution		Conc. of boscalid [mg/kg]*	
	20CRB0161-Val-high	5	2020/0390-stock		329	
	20CRB0161-Val-low	5	20CRB0161-Val-Dil2		1.54	
	20CRB0161-Val-blank	2	sample matrix		0.000	
	*Conversion from mg/L to mg/kg considering the density of ASS for diet C (1.15 g/mL)					

## Results & discussion

### Summary of method validation results:

<i>Sample description</i>	<i>Number of replicates</i>	<i>Nominal conc. of boscalid [mg/kg]</i>	<i>Mean analysed conc. of boscalid [mg/kg]</i>	<i>Mean recovery [% of nominal]</i>	<i>RSD [%]</i>
20CRB0161-Val-high	5	329	302	91.8	1.69
20CRB0161-Val-low	5	1.54	1.51	98.0	1.82
20CRB0161-Val-blank	2	0.000	< 30% LOQ	-	-

LOQ = 1.54 mg/kg of boscalid

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

The limit of quantification (LOQ) was defined in the context of this analytical phase as the lowest successfully validated fortification level (1.54 mg/kg of boscalid, corresponding to 7.72 µg/L regarding the applied dilution factor for the validation low samples).

#### *Analysis results*

<i>Sample identification</i>	<i>Nominal conc. of boscalid [mg/kg]</i>	<i>Analysed conc. of boscalid [mg/kg]</i>	<i>Recovery [% of nominal]</i>
20BLC0079-D3-AC-A	0.000	< 30% LOQ	-
20BLC0079-D4-AC-A	0.000	< 30% LOQ	-
20BLC0079-D5-AC-A	0.000	< 30% LOQ	-
20BLC0079-D6-AC-A	0.000	< 30% LOQ	-
20BLC0079-D3-AT-A	251	228	91.1
20BLC0079-D4-AT-A		227	90.4
20BLC0079-D5-AT-A		233	93.0
20BLC0079-D6-AT-A		244	97.5
20BLC0079-D3-BT-A	83.5	68.3	81.9
20BLC0079-D4-BT-A		75.8	90.8
20BLC0079-D5-BT-A		76.2	91.2
20BLC0079-D6-BT-A		79.5	95.2
20BLC0079-D3-CT-A	27.8	24.8	88.9
20BLC0079-D4-CT-A		24.9	89.5
20BLC0079-D5-CT-A		24.6	88.4
20BLC0079-D6-CT-A		26.5	95.1
20BLC0079-D3-DT-A	9.28	8.24	88.9
20BLC0079-D4-DT-A		8.44	91.0
20BLC0079-D5-DT-A		8.35	90.0
20BLC0079-D6-DT-A		8.61	92.8
20BLC0079-D3-ET-A	3.08	2.86	92.8
20BLC0079-D4-ET-A		2.57	83.2
20BLC0079-D5-ET-A		2.89	93.7
20BLC0079-D6-ET-A		2.60	84.3

LOQ = 1.54 mg/kg of boscalid

The recoveries of boscalid in the samples were between 81.9% and 97.5%. No boscalid was detected in the control samples. Thus, the concentrations of boscalid in final diets from the biological phase were verified.

### **Conclusion**

The specificity of the method was assured by multiple reaction monitoring (MRM)-detection with two transitions and the absence of interfering peaks. The ratio of quantifier and qualifier ions was recorded and was constant within  $\pm 20\%$ .

The recovery and precision data show that the influences of sample matrix were within the limits of the guidance document SANTE/2020/12830, Rev.1; all criteria were fulfilled:

- blank values did not exceed 30% of the lowest validated concentration,
- mean recoveries for each level were in the range 70-120%,
- the RSD was  $< 20\%$  per level.