

# FINAL REGISTRATION REPORT

## **Part B**

### **Section 5**

#### **Analytical Methods**

Detailed summary of the risk assessment

Product code: SIP 41061

Product name: SIP 41061

Chemical active substance:

Prothioconazole 400 g/L SC

Central Zone

Zonal Rapporteur Member State: Poland

#### **CORE ASSESSMENT**

(authorization of use)

Applicant: Sipcam Oxon S.p.A.

Submission date: April 2022

MS Finalisation date: March 2023; 06/2023

## Version history

When	What
April 2022	Applicant version submission date
02/2023	Update reference table since final report of study RAU-027-21 is now available. Changes are highlighted in <b>green</b> , old text is <del>crossed-out</del> .
03/2023	ZRM s evaluated dRR submitted by Applicant.
06/2023	RR update following commenting phase. Changes are highlighted in yellow.

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## 5 Analytical methods

### 5.1 Conclusion and summary of assessment

Submitted data are sufficient for evaluation.

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

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

Noticed data gaps are:

- Monitoring methods with lowered LOQs for high water content (in relation to use on fruits and sugar beet roots) and high starch content matrices (to comply with the lowest MRL). This data gap can be fulfilled as a post-registration requirement. The assessment should be revised when the active substance is renewed and the new methods should be provided by the applicant for re-evaluation.
- ILV method for determination prothioconazole residues in drinking water (post registration requirement).
- ILV method for the determination of residues in honey for monitoring purpose (post registration requirement).
- Extraction efficiency for the plant and animal methods (post registration requirement).

Commodity/crop	Supported/ Not supported
high water content matrices	Supported
high starch content matrices	Supported
high oil content matrices	Supported

For proposed crops please refer to Part A of the present submission.

### 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 for the analysis of Prothioconazole in plant protection product is provided as follows.

Comments of zRMS:	<b>Conclusions:</b> The analytical method HPLC/UV-DAD for determination of Prothioconazole has been submitted. The analytical method is validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev 5, therefore the method is acceptable.
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The following analytical method for the determination of Prothioconazole in SIP 41061 has not previously been reviewed and is provided in support of this assessment.

Reference:	KCP 5.1.1/01
Report	SIP41061 (PROTHIOCONAZOLE 400 g/L SC) Physical and chemical properties on fresh sample, after accelerated stability at +54°C for 14 days and after low stability at 0°C for 7 days Massardi E., 2021 Research Center BioSphereS by Biotechnologie BT Report n.: CPU-026-21
Guideline(s):	EU Regulation 1107/2009 as set out in Regulation (EU) 284/2013 and EC Guideline SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Materials and methods

The content of Prothioconazole in SIP 41061 was determined by the HPLC/UV-DAD analytical method that was validated, according to SANCO/3030/99 rev.4 (11.07.2000) Guidance Document, in the present study.

Details of the analytical method are hereunder reported.

Apparatus	
Liquid chromatograph	HPLC series 1200 with Diode Array Detector/AGILENT (code LM01)
Column	Ascentis express 150 x 4.6 mm, 2.7 µm/Supelco
Experimental conditions	
Eluent A	water + 0.1 % H <sub>3</sub> PO <sub>4</sub>
Eluent B	acetonitrile
Isocratic Mode	water + 0.1 % H <sub>3</sub> PO <sub>4</sub> : Acetonitrile = 40 : 60
Flow rate	0.800 mL/min
Injected volume	10.0 µL
Column temperature	30.0°C
Analysis wavelength	254 nm
Run time	10 minutes
Retention time prothioconazole	5.2 min approx

## Validation - Results and discussions

**Table 5.2-1: Methods suitable for the determination of Prothioconazole in plant protection product SIP 41061**

	Prothioconazole
Author, year	Massardi E., 2021
Principle of method	HPLC/UV-DAD
Linearity (linear between	linear between the range (five concentration levels): 30.1 µg/mL – 70.2 µg/mL

	Prothioconazole
mg/L / % range of the declared content) (correlation coefficient, expressed as r)	232.9 g/L – 543.3 g/L, corresponding to 20.04% – 46.7%  $R^2 = 0.99992$
Precision – Repeatability Mean (%RSD)	mean (n=5) = 41.27%, w/v, corresponding to 412.7 g/L  %RSD = 0.50% Horrat ratio < 1
Accuracy (% total Recovery)	n = 2 (low level) accuracy = 101.9%  n=2 (high level) accuracy = 101.9%
Interference/ Specificity	From the comparison of chromatograms, it is possible to affirm that the peak of active substance is well separated from the other peaks, therefore following the chromatographic conditions of method, interferences can be avoided and active substance content can be reliable determined in the test item.
Comment	-

## Conclusion

All validation parameters meet the requirements of the guidance document SANCO/3030/99 rev.5.  
The method is acceptable.

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

An overview on the acceptable methods for the analysis of relevant impurities (Prothioconazole-desthio and Toluene) of Prothioconazole in PPP is here provided.

Comments of zRMS:	<b>Conclusions:</b> The analytical method HPLC/UV-DAD for determination of Prothioconazole-desthio has been submitted. The analytical method is validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev 5, therefore the method is acceptable. The analytical method GC/MS for determination of Toluene has been submitted. According to SANCO 3030/99 rev 5 in case of relevant impurities the LOQ precision and recovery should be determined. There is no precision determined at LOQ level, however precision was determined at higher level of recovery. The value of higher level of recovery is still in acceptable limits of relevant impurity: toluene. therefore it can be concluded that the analytical method is validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev 5, therefore the method is acceptable.
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The analytical method for Prothioconazole-desthio is here summarized:

Reference:	KCP 5.1.1/02 (submitted as KCP 5.1.1/01)
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Report	SIP41061 (PROTHIOCONAZOLE 400 g/L SC) Physical and chemical properties on fresh sample, after accelerated stability at +54°C for 14 days and after low stability at 0°C for 7 days Massardi E., 2021 Research Center BioSphereS by Biotecnologie BT Report n.: CPU-026-21
Guideline(s):	EU Regulation 1107/2009 as set out in Regulation (EU) 284/2013 and EC Guideline SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Materials and methods

The content of Prothioconazole-desthio was determined by the HPLC/UV-DAD analytical method that was validated, according to SANCO/3030/99 rev.5 Guidance Document, in this study. Details of the analytical method are hereunder reported.

Apparatus	
Liquid chromatograph	HPLC series 1200 with Diode Array Detector / AGILENT (code LM01)
Liquid chromatograph-MS	HPLC 1290 Infinity with autosampler, Mass Spectrometer mod. 6490 Triple Quadrupole detector / AGILENT (code LM2)
Column	Ascentis express 150 x 4.6 mm, 2.7 µm/Supelco
Experimental conditions	
Eluent A	water + 0.1 % H <sub>3</sub> PO <sub>4</sub>
Eluent B	acetonitrile
Isocratic Mode	water + 0.1 % H <sub>3</sub> PO <sub>4</sub> : acetonitrile = 50 : 50
Diluent	acetonitrile
Flow rate	0.800 mL/min
Injected volume	3.0 µL
Column temperature	30.0 °C
Analysis wavelength	220 nm
Run time	15.0 minutes
Retention time toluene	7.1 min approx

## Validation - Results and discussions

**Table 5.2-2: Methods suitable for the determination of Prothioconazole-desthio in plant protection product (PPP) SIP 41061**

	Prothioconazole-desthio (0.17 g/kg max in SIP 41061)
Author(s), year	Massardi E., 2021
Principle of method	HPLC/UV-DAD
Linearity (linear between mg/L)	linear between the range (five concentration levels): 1.0 µg/mL – 10.0 µg/mL 0.10 g/kg – 1.0 g/kg

	<b>Prothioconazole-desthio (0.17 g/kg max in SIP 41061)</b>
<b>(correlation coefficient, expressed as r)</b>	$R^2 = 0.99997$
<b>Precision – Repeatability Mean (%RSD)</b>	mean (n=5) = 0.15 g/kg low fortification level (LOQ level) %RSD = 0.49% Horrat ratio < 1
<b>Accuracy (% total Recovery)</b>	n = 2 (low level = LOQ) accuracy = 113.0%  n=2 (high level = 0.50 g/kg) accuracy = 101.6%
<b>Interference/ Specificity</b>	A comparison between chromatograms obtained shows no interferences and all peaks are well separated from each other. Following the adjusted chromatographic conditions of methods, interferences can be avoided and the impurity content can be reliably determined in the test item
<b>LOQ</b>	0.13 g/kg
<b>Comment</b>	-

### Conclusion

All validation parameters meet the requirements of the guidance document SANCO/3030/99 rev.5.  
The method is acceptable.

The analytical method for Toluene is here summarized:

Reference:	KCP 5.1.1/03 ( <i>submitted as KCP 5.1.1/01</i> )
Report	SIP41061 (PROTHIOCONAZOLE 400 g/L SC) Physical and chemical properties on fresh sample, after accelerated stability at +54°C for 14 days and after low stability at 0°C for 7 days Massardi E., 2021 Research Center BioSphereS by Biotechnologie BT Report n.: CPU-026-21
Guideline(s):	EU Regulation 1107/2009 as set out in Regulation (EU) 284/2013 and EC Guideline SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Materials and methods

The content of Toluene was determined by the GC/MS analytical method that was validated, according to SANCO/3030/99 rev.5 Guidance Document, in this study.  
Details of the analytical method are hereunder reported.

<b>Apparatus</b>	
Liquid chromatograph	GC series Trace 1310 with Single Quadrupole Mass Detector and



	Headspace Autosampler - THERMO (code GS01)		
Column	Rxi-624Sil MS - 30 m x 0.25 mm x 1.4 µm - Restek		
Experimental conditions			
Oven temperature	Initial temperature: 35 °C 1 <sup>st</sup> ramp: 1°C/min till T = 40 °C 2 <sup>nd</sup> ramp: 10°C/min till T = 200 °C 3 <sup>rd</sup> ramp: 30°C/min till T = 290 °C, hold for 2 minutes		
Retention Time	9.8 min approx		
Injection (Headspace)	Temperature	Mode	Incubation temperature
	250°C	Split with flow 10 mL/min	100 °C
	Incubation time	Syringe temperature	Injection Volume
	30 minutes	110 °C	1 mL
Gas carrier	Helium, flow 1.7 mL/min		
Analysis time	26 minutes		
MS	Ionization mode	Polarity	Ion source temperature
	EI	Positive	300 °C
	Transfer line temperature	Scan time	-
	300 °C	0.02 s	
SIM 1 <sup>st</sup> time segment (from 0 to 16 minutes)	Quantifier	1 <sup>st</sup> Qualifier	2 <sup>nd</sup> Qualifier
	92	65	91

## Validation - Results and discussions

**Table 5.2-3: Methods suitable for the determination of Toluene in plant protection product (PPP) SIP 41061**

	<b>Toluene (1.72 g/kg max in SIP 41061)</b>
<b>Author(s), year</b>	Massardi E., 2021
<b>Principle of method</b>	GC/MS
<b>Linearity (linear between mg/L)</b>	linear between the range (five concentration levels): 1.0 µg/mL – 10.0 µg/mL 0.004 g/kg – 0.40 g/kg
<b>(correlation coefficient, expressed as r)</b>	R <sup>2</sup> = 0.99362
<b>Precision – Repeatability Mean (%RSD)</b>	mean (n = 5) = 0.049 g/kg %RSD = 2.72 % Horrat ratio < 1

	<b>Toluene</b> <b>(1.72 g/kg max in SIP 41061)</b>
<b>Accuracy</b> <b>(%total Recovery)</b>	n = 2 (low level = LOQ) accuracy = 117.0%  n=2 (high level = 0.04 g/kg) accuracy = 107.6%
<b>Interference/ Specificity</b>	A comparison between chromatograms obtained shows no interferences and all peaks are well separated from each other. Following the adjusted chromatographic conditions of methods, interferences can be avoided and the impurity content can be reliable determined in the test item
<b>LOQ</b>	0.02 g/kg
<b>Comment</b>	-

## Conclusion

All validation parameters meet the requirements of the guidance document SANCO/3030/99 rev.5.  
The method is acceptable.

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

There are no toxicologically or ecotoxicologically relevant coformulants used in the preparation Prothioconazole 400 g/L SC. However, for methods regarding determination of coformulants, reference is made to information available from the respective manufacturers.

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

No CIPAC methods for the determination of the active substance Prothioconazole are available.

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

An overview on the acceptable methods for analysis of residues of Prothioconazole for the generation of pre-authorization data is given in the following tables.

**Table 5.2-4: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Wheat grain	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2000a
	Confirmatory			
Wheat straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley grain	Primary	0.01 mg/kg	HPLC-MS/MS	

Component of residue definition: Prothioconazole					
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year	
	Confirmatory				
Barley straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS		
	Confirmatory				
Wheat grain	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2000b	
	Confirmatory				
Wheat straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS		
	Confirmatory				
Barley grain	Primary	0.01 mg/kg	HPLC-MS/MS		
	Confirmatory				
Barley straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS		
	Confirmatory				
Rape seed	Primary	0.01 mg/kg	HPLC-MS/MS		
	Confirmatory				
Rape straw, pods and green material	Primary	0.05 mg/kg	HPLC-MS/MS		
	Confirmatory				
Soil	Primary	0.006 mg/kg	HPLC – MS/MS		Schramel, 2000
	Confirmatory				
Surface and drinking water	Primary	0.1 µg/L	HPLC – MS/MS		Sommer, 2001b
	Confirmatory				
Air	Primary	1 µg/m³	HPLC – MS/MS	Maasfeld 2002	
	Confirmatory				

**Table 5.2-5: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Wheat grain	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2000a
	Confirmatory			
Wheat straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley grain	Primary	0.01 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Wheat grain	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2000b

Component of residue definition: Prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
	Confirmatory			
Wheat straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley grain	Primary	0.01 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Rape seed	Primary	0.01 mg/kg	HPLC-MS/MS	
	Confirmatory			
Rape straw, pods and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Wheat grain	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2001a
	Confirmatory			
Wheat straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley grain	Primary	0.01 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley straw and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Barley brewing malt	Primary	0.02 mg/kg	HPLC-MS/MS	
	Confirmatory			
Rape seed	Primary	0.01 mg/kg	HPLC-MS/MS	
	Confirmatory			
Rape straw, pods and green material	Primary	0.05 mg/kg	HPLC-MS/MS	
	Confirmatory			
Tomato and orange fruit	Primary	0.02 mg/kg	GC/MS	Weeren and Pelz, 2000
	Confirmatory			
Wheat grain	Primary	0.02 mg/kg	GC/MS	
	Confirmatory			
Wheat straw and green material	Primary	0.05 mg/kg	GC/MS	
	Confirmatory			
Rape seed	Primary	0.02 mg/kg	GC/MS	
	Confirmatory			
Milk, meat, offal	Primary	0.01 mg/kg	HPLC-MS/MS	.....
	Confirmatory			
Milk	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2001b

Component of residue definition: Prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
	Confirmatory			
Soil	Primary	0.006 mg/kg	HPLC – MS/MS	Schramel, 2000
	Confirmatory			
Soil	Primary	0.01 mg/kg	HPLC – MS/MS	Steinhauer, 2001
	Confirmatory			
Water	Primary	6.0 µg/L	HPLC – UV	Sommer, 1999
	Confirmatory			
Surface and drinking water	Primary	0.05 µg/L	HPLC – MS/MS	Sommer, 2001b
	Confirmatory			
Air	Primary	0.0006 µg/L	HPLC – MS/MS	Maasfeld, 2002
	Confirmatory			

**Table 5.2-6: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole-3-hydroxy-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Soil	Primary	0.005 mg/kg	HPLC – MS/MS	Schramel, 2000
	Confirmatory			
Milk, meat, offal	Primary	0.01 mg/kg	HPLC-MS/MS	.....
	Confirmatory			
Milk	Primary	0.004 mg/kg	HPLC – MS/MS	Heinemann, 2001b
	Confirmatory			

**Table 5.2-7: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole-4-hydroxy-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Milk, meat, offal	Primary	0.01 mg/kg	HPLC-MS/MS	.....
	Confirmatory			
Milk	Primary	0.004 mg/kg	HPLC – MS/MS	Heinemann, 2001b
	Confirmatory			

The following methods in crops are presented for the first time in EU among this submission; for a detailed description of them please refer to Appendix 2.

**Table 5.2-8: Validated methods for the generation of pre-authorization data**

Components of residue definition: M04, M14, M15, M16, M17 and M18				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
High water commodity (plum), high acid commodity (grape), high oil commodity (oilseed rape seeds), high protein commodity (peas dry seeds), high starch commodity (sugarbeet roots)	Primary	0.01 mg/kg	HPLC – MS/MS	Massardi, 2021
	Confirmatory			

**Table 5.2-9: Validated methods for the generation of pre-authorization data**

Component of residue definition: 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid and Triazole-lactic-acid				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
High water commodity (apple), high acid commodity (grapes), high oil commodity (oilseed rape seeds), dry commodity (peas dry seeds)	Primary	0.04 mg/kg	HPLC – MS/MS	Massardi, 2022(*)
	Confirmatory			

(\*): for the time being, only Study Plan and Draft Report are available for this study. Section B5 will be updated as soon as available Final Report. Results are in any case reported in Appendix 2

A further validation study for the determination of Prothioconazole-desthio in crops is ongoing:

Report n.: QG/20/011

Title: “Prothioconazole-desthio: Method validation in crops”

Company: Battelle UK

Section B5 will be updated as soon as available the Final Report

A description of the methods set up to support ecotoxicological and ecotoxicological studies with the determination of prothioconazole and prothioconazole-desthio in different matrices is here reported. For a detailed description of them please refer to Appendix 2.

**Table 5.2-10: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Water solutions (honeybee test medium)	Primary	10.0 µg/L	HPLC – MS/MS	Aversa, 2020
	Confirmatory			
Sugar feeding solutions (honeybee test medium)	Primary	1.0 mg/kg	HPLC – MS/MS	Aversa, 2020
	Confirmatory			

Component of residue definition: Prothioconazole				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
M4 solutions (aquatic plants and algae test media)	Primary	0.0890 mg/L	HPLC – MS/MS	Fifi A.P., 2022
	Confirmatory			
Sugar feeding solutions (honeybee test medium)	Primary	1.7533 g/kg	HPLC – MS/MS	Fifi A.P., 2022
	Confirmatory			
Soil (earthworm reproduction and growth test medium)	Primary	0.05 mg/kg	HPLC – MS/MS	Aversa, 2020
	Confirmatory			
Dislodging foliar solution	Primary	0.01 µg/mL	HPLC – MS/MS	Desiante A., 2021
	Confirmatory			

**Table 5.2-11: Validated methods for the generation of pre-authorization data**

Component of residue definition: Prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
M4 solutions (aquatic plants and algae test media)	Primary	0.1087 µg/L	HPLC – MS/MS	Fifi A.P., 2022
	Confirmatory			
Dislodging foliar solution	Primary	0.01 µg/mL	HPLC – MS/MS	Desiante A., 2021
	Confirmatory			

A further validation study for the determination of Prothioconazole-desthio and hydroxy-prothioconazole-desthio in honey is ongoing:

Report n.: QG/21/003

Title: “Magnitude of Residues of Prothioconazole-desthio and Hydroxy-prothioconazole-desthio Metabolites in Honey Following Two Tunnel Applications of a Prothioconazole 250 g/L EC Formulation (FF 065) to Phacelia in Northern and Southern Europe, 2021”

Company: Battelle UK

Section B5 will be updated as soon as available the Final Report

A further validation study for the determination of Prothioconazole-desthio and five hydroxy-prothioconazole-desthio metabolites in honey was ongoing at the time of the submission and now it is here submitted:

Report n.: QG/21/009

Title: “Prothioconazole-desthio and Hydroxy Metabolites: Method Validation in Honey”

Company: Battelle UK

**Table 5.2-11a: Validated methods for the generation of pre-authorization data**

Component of residue definition: prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio, prothioconazole-6-hydroxy-desthio, prothioconazole- $\alpha$ -hydroxy-desthio,				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Honey	Primary	0.005 mg/kg	HPLC – MS/MS	Hitchens J., 2023
	Confirmatory			

### 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)

The analytical methods for the determination of the active substance and relevant impurities in the plant protection product set out in point 5.2.1 can be applied also for post-authorization control and monitoring purpose.

#### 5.3.2 Description of analytical methods for the determination of residues of prothioconazole (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	Prothioconazole-desthio (sum of isomers)	0.02 mg/kg (LOQ) 0.01* mg/kg (LOQ) Pome fruits, Stone fruits, Cucurbits with edible peel (courgette, cucumber) and Sugar beet roots: 0.01 mg/kg Carrot and other roots and tuber vegetables (beetroots; horse radishes; parsnips; parsley roots; salsifies; swedes; turnips): 0.1 mg/kg	EFSA Scientific Report (2007) 106, 1-98  Reg. (EU) 2019/552
Plant, dry High protein/high starch		0.05 mg/kg (LOQ) 0.01* mg/kg (LOQ)	



Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
content (dry)		Wheat, rye, triticale, barley: 0.05 mg/kg	
Plant, high acid content		0.02 mg/kg (LOQ) 0.01* mg/kg (LOQ)	
Plant, high starch content		0.02 mg/kg (LOQ) 0.01* mg/kg (LOQ)	
Plant, high oil content		0.02 mg/kg (LOQ) Oilseed rape: 0.15 mg/kg	
Milk, muscle, eggs, fat	Prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio	0.004 mg/kg (LOQ) 0.01* mg/kg (LOQ)	EFSA Scientific Report (2007) 106, 1-98 Reg. (EU) 2019/552
Meat, liver, kidney, fat	Prothioconazole-desthio (sum of isomers)	0.01 mg/kg (LOQ) 0.1 mg/kg	
Soil (Ecotoxicology)	Prothioconazole, prothioconazole-desthio	0.006 mg/kg (LOQ)	EFSA Scientific Report (2007) 106, 1-98
	Prothioconazole-desthio	0.01 mg/kg (LOQ)	EFSA Scientific Report (2007) 106, 1-98
Surface and drinking water (Human toxicology)	Prothioconazole	0.1 µg/L	EFSA Scientific Report (2007) 106, 1-98
	Prothioconazole-desthio	0.05 µg/L	
Air	Prothioconazole	0.015 mg/m <sup>3</sup>	EFSA Scientific Report (2007) 106, 1-98
	Prothioconazole-desthio	0.0006 mg/m <sup>3</sup>	
Tissue (meat or liver)	-	-	open
Body fluids			

\*-LOQ

### 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 for the analysis of prothioconazole metabolite in plant matrices is given in the following tables.

zRMS:

Proposed uses belong to the high water, high starch and high oil content matrices.

According to SANTE/2020/12830 rev.1 monitoring methods have to comply with the lowest MRL for each matrix group. Since many MRLs have been lowered to 0.01 mg/kg, the validated LOQs of the EU agreed methods for high water content and high starch content matrices are not sufficient to monitor these lowered MRLs for food of plant origin.

However, it should be noted that proposed uses in cereals group are only: wheat (soft, durum), triticale, rye and barley. MRLs for these crops are higher. Therefore presented in this application methods are sufficient to monitor residues in these crops.

Methods for high oil content matrices have acceptable LOQs.

Data gaps: Methods with lowered LOQs for high water content in relation to use on fruits and sugar beet roots and high starch content matrices.

This data gap can be fulfilled as a post-registration requirement.

The assessment should be revised when the active substance is renewed and the new methods should be provided by the applicant for re-evaluation.

A further validation study for the determination of Prothioconazole-desthio and five hydroxy-prothioconazole-desthio metabolites in honey was ongoing at the time of the submission and now it is submitted:

Report n.: QG/21/009

Title: "Prothioconazole-desthio and Hydroxy Metabolites: Method Validation in Honey"

Company: Battelle UK

Method is acceptable for monitoring purpose. ILV is required.

**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: prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Dry commodity (wheat and barley straw)	Primary	0.05 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98
High starch commodity (wheat and barley grain)	Primary	0.02 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98
	ILV			
High oil commodity (canola seed)	Primary	0.02 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98
High water commodity (tomato)	Primary	0.02 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98
	ILV			
High acid commodity (orange fruit)	Primary	0.02 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98
Component of residue definition: prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio, prothioconazole-6-hydroxy-desthio, prothioconazole- $\alpha$ -hydroxy-desthio,				
Matrix type	Method type	Method LOQ	Principle of method	Author(s), year
Honey	Primary	0.005 mg/kg	HPLC – MS/MS	Hitchens J., 2023
	Confirmatory			

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

zRMS

data gap: Statement on extraction efficiency is required.

	Method for products of plant origin
Required, available from:	-
Not required, because:	no residues $\geq$ LOQ are expected

### 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 prothioconazole metabolites in animal matrices is given in the following tables.

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

Component of residue definition: prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Milk	Primary	0.004 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
	ILV			
Meat	Primary	0.01 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
Liver				
Kidney	ILV			
Fat				

**Table 5.3-5: Validated methods for food and feed of animal origin**

Component of residue definition: prothioconazole-3-hydroxy-desthio				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Milk	Primary	0.004 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
	ILV			
Meat	Primary	0.01 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
Liver				
Kidney	ILV			
Fat				

**Table 5.3-6: Validated methods for food and feed of animal origin**

Component of residue definition: prothioconazole-4-hydroxy-desthio				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Milk	Primary	0.004 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
	ILV			
Meat	Primary	0.01 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98
Liver				
Kidney	ILV			
Fat				

**Table 5.3-7: Statement on extraction efficiency**

zRMS

data gap: Statement on extraction efficiency is required.

	Method for products of animal origin
Required, available from:	-
Not required, because:	no residues $\geq$ LOQ are expected

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole and prothioconazole-desthio in soil is given in the following tables.

**Table 5.3-8: Validated methods for soil**

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	Method LOQ	Principle of method	EU agreed
Primary	0.006 mg/kg	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98

**Table 5.3-9: Validated methods for soil**

Component of residue definition: prothioconazole-desthio			
Method type	Method LOQ	Principle of method	EU agreed
Primary	0.01 mg/kg	GC-MS	EFSA Scientific Report (2007) 106, 1-98

#### 5.3.2.4 Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole and its metabolite in surface and drinking water is given in the following tables.

zRMS

data gap: ILV method for determination prothioconazole residues in drinking water.

NL comment: *The residue definition is Prothioconazole and Prothioconazole-desthio. For both these components a method is available with an LOQ  $\geq$  0.1  $\mu$ g/L to comply with the Dutch drinking water decree (Drinkwaterbesluit)*

**Table 5.3-10: Validated methods for water**

Component of residue definition: prothioconazole				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Surface, ground and tap water	Primary	0.1 $\mu$ g/L	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98

**Table 5.3-11: Validated methods for water**

Component of residue definition: prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method	EU agreed
Surface, ground and tap water	Primary	0.05 µg/L	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98

### 5.3.2.5 Description of methods for the analysis of air (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole and its metabolite in air is given in the following tables.

**Table 5.3-12: Validated methods for air**

Component of residue definition: prothioconazole			
Method type	Method LOQ	Principle of method	EU agreed
Primary	0.015 mg/m <sup>3</sup>	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98

**Table 5.3-13: Validated methods for air**

Component of residue definition: prothioconazole-desthio			
Method type	Method LOQ	Principle of method	EU agreed
Primary	0.0006 mg/m <sup>3</sup>	HPLC-MS/MS	EFSA Scientific Report (2007) 106, 1-98

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

Not available.

zRMS:

According to the Regulation (EU) No 284/2013 method for the determination of residues in body fluids and tissue is required. Currently agreed EU endpoints for prothioconazole do not include a residue definition for monitoring in body fluids and tissues.

The development of monitoring methods for body fluids and tissues will be required once the active substance is renewed and the residue definitions in these matrices are finalized at EU level.

### 5.3.2.7 Other studies/ information

No other studies have been reported in the present submission.

## 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 5.1.1/01 KCP 5.1.1/02  [submitted as KCP 2.1/01 in B1, B2 and B5]	Massardi E.	2021	SIP41061 (PROTHIOCONAZOLE 400 g/L SC) Physical and chemical properties on fresh sample, after accelerated stability at +54°C for 14 days and after low stability at 0°C for 7 days CPU-026-21 Research Center BioSpheres by Biotechnologie BT GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/01	Massardi E.	2021	Validation of the analytical method to determine prothioconazole metabolites in high water commodity (plum), high acid commodity (grape), high oil commodity (oilseed rape seeds), high protein commodity (peas dry seeds) and high starch commodity (sugar beet roots) RAU-003-21 Research Center BioSphereS by Biotechnologie BT GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/02	Massardi E.	2022	Validation of the analytical method to determine Triazole Derivative Metabolites (TDMs) in high water commodity (zucchini), high acid commodity (grapes), oil commodity (oilseed rape seeds) and dry commodity (peas dry seeds) — <del>amended 1</del> <b>FINAL REPORT</b> Study Plan RAU-027-21 Research Center BioSphereS by Biotechnologie BT GLP not published	N	Sipcam Oxon S.p.A.

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP 5.1.2/03	Aversa S.	2020	Validation of an HPLC-MS/MS analytical method for the determination of Prothioconazole and Azoxystrobin in water and sugar feeding solutions coming from ecotox laboratory tests (honeybees) BT214/20 Biotecnologie BT GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/04	Fifi A. P.	2022	Validation of the analytical method (SANTE/2020/12830 Rev.1) for the determination of Prothioconazole and Prothioconazole-desthio in aqueous matrix and sugar feeding solutions with product SIP 41061 BT193/21 Biotecnologie BT GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/05	Aversa S.	2020	Validation of an HPLC-MS/MS analytical method for the determination of Prothioconazole in soil coming from ecotox laboratory tests (earthworms) BT215/20 Biotecnologie BT GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/06	Desiante A.	2021	Determination of dislodgeable foliar residue of prothioconazole and prothioconazole-desthio in raw agricultural commodity peach followin two applications of SIP41061 (Prothioconazole 400 g/L SC) BIU-011-21 Research Center BioSphereS GLP not published	N	Sipcam Oxon S.p.A.
KCP 5.1.2/18	Hitchens J.	2023	Prothioconazole-desthio and hydroxy metabolites: method validation in honey QG/21/009 Battelle UK Ltd. GLP not published	N	Sipcam Oxon S.p.A.

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

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP 5.1.2/07	Heinemann O.	2000a	Analytical determination of residues of JAU 6476 and desthio-JAU 6476 in/on cereals by HPLC/MS/MS 00598 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/08	Heinemann O.	2000b	Analytical determination of residues of JAU 6476 and desthio-JAU 6476 in/on cereals and canola by HPLC/MS/MS (method modification 00598/M001) 00598/M001 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/09	Schramel O.	2000	Residue analytical method 00610 (MR-643/99) for the determination of JAU 6476 and the metabolites JAU 6476-desthio and JAU 6476-S-methyl in soil by HPLC/MS/MS 00610 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/10	Sommer H.	2001b	Enforcement method 00684 for determination of JAU 6476 and JAU 6476-desthio in drinking and surface water by HPLC/MS/MS 00684 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/11	Maasfeld W.	2002	Method for the determination of JAU 6476 in air by HPLC/MS/MS 00724 Bayer AG GLP	N	Bayer AG



<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
			published		
KCP 5.1.2/12	Heinemann O.	2001a	Analytical determination of residues of JAU6476-sulfonic acid and JAU6476-desthio in/on cereals and canola by HPLC/MS/MS (method modification 00598/M001) 00647 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/13	Weeren R.D., Pelz S.	2000	Modification M033 of method 00086: validation of DFG method S 19 (extended revision) for the determination of residues of JAU 6476-desthio in materials of plant and animal origin 00684 Bayer AG GLP published	N	Bayer AG
KCP 5.1.2/14	...	2001b	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio and JAU6476-desthio in/on matrices of animal origin by HPLC/MS/MS ... GLP published		Bayer AG
KCP 5.1.2/15	Heinemann O.	2001b	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio and JAU6476-desthio in milk by HPLC/MS/MS 00655/M001 Bayer AG GLP published	N	Bayer AG

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP 5.1.2/16	Steinhauer S.	2001	Enforcement method 00086/M038 for the determination of the residues of JAU 6476-desthio in soil - : validation of DFG method S 19 (extended revision) 00086/M038 Dr. Specht&Partner GLP published	N	Bayer AG
KCP 5.1.2/17	Sommer H.	1999	Method for the determination of JAU6476 in test water from aquatic toxicity tests by HPLC [Tox/Ecotox method] 00699 Bayer AG GLP published	N	Bayer AG

The following tables are to be completed by MS

**List of data submitted by the applicant and not relied on**

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP XX	Author	YYYY	Title Company Report N Source GLP/non GLP/GEP/non GEP Published/Unpublished	Y/N	Owner

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

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

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP XX	Author	YYYY	Title Company Report N Source GLP/non GLP/GEP/non GEP Published/Unpublished	Y/N	Owner

## Appendix 2 Detailed evaluation of submitted analytical methods

### A 2.1 Analytical methods for Prothioconazole and its metabolites

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

##### A 2.1.1.1 Analytical method 1

##### A 2.1.1.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/01

Report Validation of the analytical method to determine prothioconazole metabolites in high water commodity (plum), high acid commodity (grape), high oil commodity (oilseed rape seeds), high protein commodity (peas dry seeds) and high starch commodity (sugar beet roots)  
Massardi E., 2021  
RAU-003-21

Guidelines: SANCO/3029/99 rev. 4 (11/07/2000)\*  
SANCO/825/00 rev.8.1 (16/11/2010)\*

Deviations: No

GLP: Yes

Acceptability: Yes

*\*: the analytical method here described was validated according to SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 guidance documents, that were in place when Study Plan has been finalized Starting from the 1<sup>st</sup> March 2021 both the guidelines have been replaced by SANTE/2020/12830, rev.1 (24/02/2021) guideline but comparing the requirements and acceptability criteria of the new guideline and those related to the old ones adopted in this study, the analytical method validated is compliant also with the new guidance document, in particular for what concerns the assignment of crops groups, as here explained:*

- dry commodities group is covered by peas dry seeds matrix (previously identified as high protein);
- high water content commodities group is covered by plum and sugar beet roots matrices (the last previously identified as high starch);
- high oil content commodities group is covered by oilseed rape seeds matrix;
- high acid content commodities group is covered by grape matrix.

*In any case, all the required groups of commodities are covered*

### Materials and methods

The method consists in extraction using acetonitrile and purification by Dispersive Solid Phase Extraction (D-SPE). The purified samples were finally analyzed with a HPLC system coupled with a Triple Quadrupole Mass analyzer (LC-MS/MS).

### Sample preparation

Homogeneous sub-samples were prepared for analysis according to the Standard Operating Procedure MNG018, last version, of the Residue Analysis Unit.

The specimens were removed from storage at approximately  $-18^{\circ}\text{C}$  and homogenised by vegetable grinder (Plum, Sugar beet roots and Grape samples) or mixer (Oilseed rape seeds and Peas dry seeds samples) in order to obtain a homogeneous sample. When the analysis was not performed immediately, the homogenised samples were stored at  $-18^{\circ}\text{C}$  or lower, in a tightly-closed plastic flask.

Extraction and purification procedures are below described.

For plum, grape and sugar beet roots samples, a portion of homogeneous sample ( $5 \pm 0.03$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. The sample was extracted with 10 mL of acetonitrile by manual vigorous shaking for 2 minutes. After addition of QuEChERS Extraction kit, the sample was re-extracted by manual vigorous shaking for 2 minutes and then centrifuged at 4000/5000 rpm for 5 minutes. Then, an aliquot of 6 mL of acetonitrilic phase was taken and purified with 900 mg of magnesium sulphate anhydrous and 150 mg of Boundesil PSA resin by manual vigorous shaking for 1 minute and sequentially centrifugation at 4000/5000 rpm for 5 minutes. An aliquot of about 1.5 mL of the extract was centrifuged again with microcentrifuge at 14000 rpm for 5 minutes. The sample was finally ready to be analysed.

For oilseed rape seeds samples, a portion of homogeneous sample ( $5 \pm 0.03$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. 8.5 mL of water and 10 mL of acetonitrile were added and the sample was extracted by manual vigorous shaking for 2 minutes. After addition of 6.0 g of magnesium sulphate anhydrous and 1.5 g of sodium chloride, the sample was re-extracted by manual vigorous shaking for 2 minutes and then centrifuged at 4000/5000 rpm for 5 minutes.

Then, an aliquot of 8 mL of acetonitrilic phase was taken and purified with 1600 mg of magnesium sulphate anhydrous and 240 mg of Discovery DSC C18 by manual vigorous shaking for 1 minute and sequentially centrifugation at 4000/5000 rpm for 5 minutes. An aliquot of about 1.5 mL of the extract was centrifuged again with microcentrifuge at 14000 rpm for 5 minutes. The sample was finally ready to be analysed.

For peas dry seeds samples, a portion of homogeneous sample ( $2 \pm 0.01$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. 8.5 mL of water and 20 mL of acetonitrile were added and the sample was extracted by manual vigorous shaking for 2 minutes. After addition of QuEChERS Extraction kit, the sample was re-extracted by manual vigorous shaking for 2 minutes and then centrifuged at 4000/5000 rpm for 5 minutes.

Then, an aliquot of 6 mL of acetonitrilic phase was taken and purified with 900 mg of magnesium sulphate anhydrous and 150 mg of Boundesil PSA resin by manual vigorous shaking for 1 minute and sequentially centrifugation at 4000/5000 rpm for 5 minutes. An aliquot of about 1.5 mL of the extract was centrifuged again with microcentrifuge at 14000 rpm for 5 minutes. The sample was finally ready to be analysed.

### Apparatus description

<i>HPLC</i>	HPLC 1290 Infinity Agilent Mass spectrometer mod. 6490 Triple Quadrupole Agilent with autosampler (Code LM2)	
<i>Column</i>	Kinetex PFP, 2.6 $\mu\text{m}$ , 100 Å, 2.10 x 100 mm - Phenomenex	
<i>Guard Column*</i>	SecurityGuard Cartridge kit: C18, 4 x 3.0 mm - Phenomenex	
<i>Column temperature</i>	35°C	
<i>Injection volume</i>	2 $\mu\text{L}$	
<i>Eluent A</i>	Water + 10 mM ammonium formate	
<i>Eluent B</i>	Methanol	
<i>Retention time</i>	M18 $\approx$ 4.2 min (Peak I), 5.4 min (Peak II)	M16 $\approx$ 8.1 min

	M14 ≈ 6.0 min M15 ≈ 7.7 min		M04 ≈ 12.0 min M17 ≈ 12.5 min	
Gradient elution	Time (min)	A %	B %	Flow (mL/min)
	0	70	30	0.25
	1	48	52	0.25
	10	45	55	0.25
	13	20	80	0.25
	14	20	80	0.25
	14.1	70	30	0.25
	17	70	30	0.25
Column flow rate	0.25 mL/min			
Run time	17.0 minutes			

Analyte	Acquiring method				ESI – positive MRM	
	Parent ion (m/z)	Product ion (m/z)	CE	Dwell	Transition ID	Attribute
M04	312	70	35	50	M04	Quantifier ion
		125	35	50	M04	Qualifier ion
M14	328	70	30	50	M14	Quantifier ion
		141	30	50	M14	Qualifier ion
M15	328	70	30	50	M15	Quantifier ion
		141	30	50	M15	Qualifier ion
M16	328	70	35	50	M16	Quantifier ion
		141	35	50	M16	Qualifier ion
M17	328	70	40	50	M17	Quantifier ion
		141	40	50	M17	Qualifier ion
M18	328	70	30	50	Peak I: M18(1) Peak II: M18(2)	Quantifier ion
		141	30	50	Peak I: M18(1) Peak II: M18(2)	Qualifier ion

#### Validation data

The analytical method was validated in terms of Accuracy, Precision, Linearity, Selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANCO/825/00 rev. 8.1 (16/11/2010) and SANCO/3029/99, rev. 4 (11/07/2000) by means of recovery test and analysis of blank samples. Untreated samples were used for recovery test.

The following samples were extracted and analysed for each matrix:

Samples	Fortification level (mg/kg)	Replicates in primary detection	Replicates confirmatory detection
control	-	2	2
fortified	0.01	5	5
fortified	1.0	5	-

## Results and discussions

In the following tables, a summary of validation data obtained in primary and confirmatory detection is reported.

### Accuracy (as recovery) and precision (as repeatability)

A good accuracy was observed for all the fortified specimens analysed. All the recoveries were within the acceptable range of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was well below 20%.

**Table A 1: Recovery results from method validation of M04, M14, M15, M16, M17 and M18 using the analytical method in primary detection**

Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5	Overall recovery (%) n=10	RSD (%) n=10
Plum	M04	0.01	87.91	2.42	90.48	3.99
		1.0	93.05	3.10		
	M14	0.01	86.05	1.54	91.61	6.51
		1.0	97.17	1.08		
	M15	0.01	82.72	1.72	89.92	8.54
		1.0	97.11	1.20		
	M16	0.01	83.86	2.61	91.04	8.51
		1.0	98.22	1.24		
	M17	0.01	90.09	6.71	91.49	4.70
		1.0	92.88	0.54		
	M18	0.01	86.19	1.49	90.57	5.32
		1.0	94.96	1.69		
Oilseeds rape seeds	M04	0.01	78.31	1.64	83.79	7.25
		1.0	89.27	2.81		
	M14	0.01	82.37	11.38	87.99	9.89
		1.0	93.61	2.07		
	M15	0.01	77.99	6.67	78.50	5.09
		1.0	79.02	3.61		
	M16	0.01	95.65	6.03	100.62	7.13
		1.0	105.58	4.34		
	M17	0.01	83.71	3.73	92.67	10.55
		1.0	101.63	2.13		
	M18	0.01	88.40	2.12	88.96	2.34
		1.0	89.51	2.60		

Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5	Overall recovery (%) n=10	RSD (%) n=10
Peas dry seeds	M04	0.01	89.95	6.37	98.72	10.28
		1.0	107.50	2.36		
	M14	0.01	103.72	3.62	100.57	4.28
		1.0	97.43	1.71		
	M15	0.01	104.43	4.00	100.12	5.81
		1.0	95.81	3.66		
	M16	0.01	102.96	3.93	100.55	4.27
		1.0	98.14	3.31		
	M17	0.01	73.87	4.31	85.22	14.35
		1.0	96.57	2.09		
	M18	0.01	101.60	3.71	99.92	3.31
		1.0	98.24	1.90		
Grape	M04	0.01	82.0	8.46	94.51	4.83
		1.0	99.63	11.93		
	M14	0.01	95.30	4.56	94.85	3.45
		1.0	97.18	5.77		
	M15	0.01	88.80	4.23	91.79	12.07
		1.0	91.52	7.43		
	M16	0.01	100.11	1.04	99.49	5.38
		1.0	98.88	8.00		
	M17	0.01	93.99	5.63	95.71	5.37
		1.0	97.43	5.04		
	M18	0.01	91.68	1.23	90.55	3.08
		1.0	89.42	4.04		
Sugar beet roots	M04	0.01	86.56	0.45	89.33	3.64
		1.0	92.10	2.29		
	M14	0.01	93.10	6.93	92.75	6.74
		1.0	92.40	7.34		
	M15	0.01	89.28	6.50	89.60	7.90
		1.0	89.92	9.88		
	M16	0.01	91.48	5.69	89.53	8.81
		1.0	87.58	11.61		
	M17	0.01	84.73	4.02	86.30	5.81
		1.0	87.87	7.09		



Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5	Overall recovery (%) n=10	RSD (%) n=10
	M18	0.01	89.92	6.05	89.69	5.96
		1.0	89.46	6.58		

**Table A 2: Recovery results from method validation of M04, M14, M15, M16, M17 and M18 using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5
Plum	M04	0.01	85.72	2.14
	M14	0.01	83.74	1.22
	M15	0.01	81.84	1.86
	M16	0.01	86.62	2.63
	M17	0.01	89.56	6.85
	M18	0.01	89.20	1.78
Oilseed rape seeds	M04	0.01	78.83	1.38
	M14	0.01	88.34	12.04
	M15	0.01	86.73	7.75
	M16	0.01	92.29	5.87
	M17	0.01	86.58	3.71
	M18	0.01	102.43	3.74
Peas dry seeds	M04	0.01	93.64	6.03
	M14	0.01	101.76	4.54
	M15	0.01	105.05	4.17
	M16	0.01	103.06	4.39
	M17	0.01	71.06	6.02
	M18	0.01	91.10	5.51
Grape	M04	0.01	95.04	2.47
	M14	0.01	93.45	0.94
	M15	0.01	81.01	4.25
	M16	0.01	104.22	2.06
	M17	0.01	95.31	5.53
	M18	0.01	93.65	1.18
Sugar beet roots	M04	0.01	89.97	1.75

<b>Matrix</b>	<b>Analyte</b>	<b>Fortification level (mg/kg) n = 5</b>	<b>Mean recovery (%) n=5</b>	<b>RSD (%) n=5</b>
	M14	0.01	93.21	7.16
	M15	0.01	89.10	6.69
	M16	0.01	95.58	5.37
	M17	0.01	84.45	3.94
	M18	0.01	92.26	5.43

**Table A 3: Characteristics of the analytical method used for the validation of M04, M14, M15, M16, M17 and M18 residues in plum, oilseed rape seeds, peas dry seeds, grape, and sugar beet roots**

	M04		M14		M15		M16		M17		M18		
Specificity	MS spectra provided; blank value < 30 % LOQ for all matrices												
Calibration (matrix matched standard solutions at five concentration levels for all matrices)	plum												
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	
	y = 55487x + 3897 R <sup>2</sup> > 0.99	y = 19694x + 2233 R <sup>2</sup> > 1.0	y = 144372x – 16093 R <sup>2</sup> > 0.99	y = 35061x + 63 R <sup>2</sup> > 0.99	y = 146717x + 15879 R <sup>2</sup> > 0.99	y = 63038x + 6187 R <sup>2</sup> > 0.99	y = 129522x + 8650 R <sup>2</sup> > 0.99	y = 30909x – 3977 R <sup>2</sup> > 0.99	y = 156761x – 39446 R <sup>2</sup> > 0.99	y = 47467x – 14941 R <sup>2</sup> > 0.99	y = 137437x – 4933 R <sup>2</sup> > 0.99	y = 11480x – 2031 R <sup>2</sup> > 0.99	
	oilseed rape seeds												
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	
	y = 37741x + 15556 R <sup>2</sup> > 0.99	y = 13804x + 3881 R <sup>2</sup> > 0.99	y = 40475x + 2210 R <sup>2</sup> > 0.99	y = 9830x – 1269 R <sup>2</sup> > 0.99	y = 61170x + 36627 R <sup>2</sup> > 0.99	y = 28312x + 1382 R <sup>2</sup> > 0.99	y = 73712x – 23948 R <sup>2</sup> > 0.99	y = 17972x – 1259 R <sup>2</sup> > 0.99	y = 114209x + 38743 R <sup>2</sup> > 0.99	y = 36212x + 5611 R <sup>2</sup> > 0.99	y = 27020x – 3750 R <sup>2</sup> > 0.99	y = 2112x – 1572 R <sup>2</sup> > 0.99	
	peas dry seeds												
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 43869x + 7727 R <sup>2</sup> > 0.99	y = 15649x + 2901 R <sup>2</sup> > 0.99	y = 91753x – 5899 R <sup>2</sup> > 0.99	y = 22402x – 620 R <sup>2</sup> > 0.99	y = 98629x – 10818 R <sup>2</sup> > 0.99	y = 42026x – 2432 R <sup>2</sup> > 0.99	y = 100422x – 4012 R <sup>2</sup> > 0.99	y = 23932x + 586 R <sup>2</sup> > 0.99	y = 80549x + 16244 R <sup>2</sup> > 0.99	y = 24155x + 7842 R <sup>2</sup> > 0.99	y = 111353x – 7032 R <sup>2</sup> > 0.99	y = 9474x + 1428 R <sup>2</sup> > 0.99	

	M04		M14		M15		M16		M17		M18	
	grape											
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 41157x + 9198 R <sup>2</sup> > 0.99	y = 14655x R <sup>2</sup> > 0.99	y = 292348x – 156709 R <sup>2</sup> > 0.99	y = 70469x – 32040 R <sup>2</sup> > 0.99	y = 354592x + 131273 R <sup>2</sup> > 0.99	y = 155697x + 79972 R <sup>2</sup> > 0.99	y = 416956x + 16166 R <sup>2</sup> > 0.99	y = 102600x – 26456 R <sup>2</sup> > 0.99	y = 267841x + 47876 R <sup>2</sup> > 0.99	y = 80657x + 6899 R <sup>2</sup> > 0.99	y = 254794x + 20114 R <sup>2</sup> > 0.99	y = 21062x – 2717 R <sup>2</sup> > 0.99
	sugar beet roots											
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 48114x + 4753 R <sup>2</sup> > 0.99	y = 17143x – 1199 R <sup>2</sup> > 0.99	y = 288988x – 17571 R <sup>2</sup> > 0.99	y = 71009x – 6793 R <sup>2</sup> > 0.99	y = 294185x – 30428 R <sup>2</sup> > 0.99	y = 127588x – 19153 R <sup>2</sup> > 0.99	y = 269572x – 86763 R <sup>2</sup> > 0.99	y = 64389x – 32058 R <sup>2</sup> > 0.99	y = 185292x + 633 R <sup>2</sup> > 0.99	y = 55951x – 5522 R <sup>2</sup> > 0.99	y = 241006x – 17497 R <sup>2</sup> > 0.99	y = 19904x – 5609 R <sup>2</sup> > 0.99
Calibration range	For plum, grape, oilseeds rape seeds and sugar beet roots from 0.0015 µg/mL to 0.10 µg/mL, corresponding in matrix from 0.003 mg/kg to 0.20 mg/kg and 2 mg/kg (*); for peas dry seeds from 0.0003 µg/mL to 0.02 µg/mL, corresponding in matrix from 0.003 mg/kg to 0.20 mg/kg and 2 mg/kg (*). (*): this data is obtained considering the maximum dilution factor applied to samples during analytical sequences (5 g of sample to 100 mL), done to include in the linearity range the recovery tests carried out at 1.0 mg/kg Concentration ranges covered from 30% of the LOQ to 20% above the highest measured concentration											
Assessment of matrix effects is presented	yes											

	<b>M04</b>	<b>M14</b>	<b>M15</b>	<b>M16</b>	<b>M17</b>	<b>M18</b>
Limit of quantification (LOQ)	0.01 mg/kg (for all matrices)					
Limit of detection (LOD)	0.003 mg/kg (for all matrices)					

## Conclusion

The analytical method was suitable to determine residues of M04, M14, M15, M16, M17 and M18 in plum, oilseed rape seeds, peas dry seeds, grape, and sugar beet roots. All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied for both primary and confirmatory detection.

Limit of quantification (LOQ) was successfully assessed to 0.01 mg/kg in all matrices and for all analyzed compounds.

### A 2.1.1.1.2 Analytical method 2

#### A 2.1.1.1.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/02

Report Validation of the analytical method to determine Triazole Derivative Metabolites (TDMs) in high water commodity (zucchini), high acid commodity (grapes), oil commodity (oilseed rape seeds) and dry commodity (peas dry seeds) – ~~amended 1 (SP)~~  
Massardi E., 2022  
RAU-027-21

Guidelines: SANTE/2020/12830, rev.1 (24/02/2021)\*

Deviations: No

GLP: Yes

Acceptability: Yes

~~\*: since the Final Report of this study is not available, in the present submission results of the Draft Report are presented. Study Plan is here submitted as doc K and Final Report will be presented furtherly.~~

## Materials and methods

The method consists in extraction using methanol with 1% formic acid and, when necessary, a purification step by C18-sorbent (Discovery DSC-18). The extracted samples were finally analyzed with a HPLC system coupled with a Triple Quadrupole Mass analyzer with Differential Mobility Spectrometry (LC-DMS/MS/MS).

### Sample preparation

Homogeneous sub-samples were prepared for analysis according to the Standard Operating Procedure MNG018, last version, of the Residue Analysis Unit.

The specimens were removed from storage at approximately –18°C and homogenised by vegetable grinder (Apple and Grape samples) or mixer (Oilseed rape seeds and Peas dry seeds samples) in order to obtain a homogeneous sample. When the analysis was not performed immediately, the homogenised samples were stored at –18°C or lower, in a tightly-closed plastic flask.

Extraction and purification procedures are below described.

For apple and grape samples, a portion of homogeneous sample ( $10 \pm 0.05$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. The sample was extracted with 10 mL of methanol with 1 % of formic acid by manual vigorous shaking for 3 minutes. After centrifugation at 4000/5000 rpm for 5 minutes, the sample was placed in the freezer at  $-18^{\circ}\text{C}$  for at least one hour. Then the extract was filtered on a  $0.2\ \mu\text{m}$  RC filter and was finally ready to be analysed.

For oilseed rape seeds samples, a portion of homogeneous sample ( $5 \pm 0.03$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. 10 mL of water and 10 mL of hexane were added and the sample was extracted by manual vigorous shaking for 3 minutes. After centrifugation at 4000/5000 rpm for 5 minutes, the higher hexane phase was eliminated. The sample was re-extracted with 10 mL of methanol with 1 % of formic acid by manual vigorous shaking for 3 minutes and then centrifuged at 4000/5000 rpm for 5 minutes. The extract was placed in the freezer at  $-18^{\circ}\text{C}$  for at least one hour.

Then, an aliquot of 4 mL of methanolic phase was taken and purified with 200 mg of Discovery DSC C18 by manual vigorous shaking for 1 minute and sequentially centrifugation at 4000/5000 rpm for 5 minutes. Then the extract was filtered on a  $0.2\ \mu\text{m}$  RC filter and was finally ready to be analysed.

For peas dry seeds samples, a portion of homogeneous sample ( $5 \pm 0.03$  g) was weighed into a 50 mL centrifuge tube; recovery samples were spiked at this point. 10 mL of water and 10 mL of methanol with 1 % of formic acid were added and the sample was extracted by manual vigorous shaking for 3 minutes. After centrifugation at 4000/5000 rpm for 5 minutes, the sample was placed in the freezer at  $-18^{\circ}\text{C}$  for at least one hour.

Then, an aliquot of 4 mL of methanolic phase was taken and purified with 200 mg of Discovery DSC C18 by manual vigorous shaking for 1 minute and sequentially centrifugation at 4000/5000 rpm for 5 minutes. Then the extract was filtered on a  $0.2\ \mu\text{m}$  RC filter and was finally ready to be analysed

#### Apparatus description

<i>HPLC</i>	HPLC 1290 Infinity II Agilent/ Mass spectrometer mod. Q-Trap 5500 Triple Quadrupole with SelexION Differential Mobility Spectrometry ABSciex (Code LM3)			
<i>Column</i>	Hypercarb, 5 μm, 100 x 2.1 mm – Thermo scientific			
<i>Column temperature</i>	40°C			
<i>Injection volume</i>	2 μL			
<i>Eluent A</i>	1% acetic acid in water/methanol 95/5			
<i>Eluent B</i>	1% acetic acid in methanol			
<i>Retention time</i>	1,2,4-Triazole ≈ 0.9 min Triazole-alanine ≈ 1.1 min Triazole-lactic-acid ≈ 2.6 min Triazole-acetic acid ≈ 2.9 min			
<i>Gradient elution</i>	<b>Time (min)</b>	<b>A %</b>	<b>B %</b>	<b>Flow (mL/min)</b>
	0	100	0	0.6
	5	10	90	
	6	10	90	
	6.1	100	0	
	10	100	0	
<i>Column flow rate</i>	0.6 mL/min			
<i>Run time</i>	10.0 minutes			

Analyte	Acquiring method			ESI – positive MRM	
	Parent ion (m/z)	Product ion (m/z)	CE	Transition ID	Attribute
1,2,4-Triazole	70	43	30	1,2,4-Triazole - 1	Quantifier ion
		70	15	1,2,4-Triazole - 2	Qualifier ion
Triazole alanine	157	70	18	Triazole alanine - 1	Quantifier ion
		88	17	Triazole alanine - 2	Qualifier ion
Triazole acetic acid	128	70	25	Triazole acetic acid - 1	Quantifier ion
		43	55	Triazole acetic acid - 1	Qualifier ion
Triazole lactic acid	158	70	25	Triazole lactic acid - 1	Quantifier ion
		43	60	Triazole lactic acid - 2	Qualifier ion

#### Validation data

The analytical method was validated in terms of Accuracy, Precision, Linearity, Selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANTE/2020/12830, rev.1 (24/02/2021) by means of recovery test and analysis of blank samples. Untreated samples were used for recovery test.

The following samples were extracted and analysed for each matrix:

Samples	Fortification level (mg/kg)	Replicates in primary detection	Replicates confirmatory detection
control	-	2	2
fortified	0.04	5	5
fortified	1.0	5	-

#### Results and discussions

In the following tables, a summary of validation data obtained in primary and confirmatory detection is reported.

#### Accuracy (as recovery) and precision (as repeatability)

A good accuracy was observed for all the fortified specimens analysed. All the recoveries were within the acceptable range of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was well below 20%.

**Table A 4: Recovery results from method validation of 1,2,4-Triazole, Triazole alanine, Triazole acetic acid and Triazole lactic acid using the analytical method in primary detection**

Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5	Overall recovery (%) n=10	RSD (%) n=10
Apple	1,2,4-Triazole	0.04	101.83	6.81	98.12	7.08
		1.0	94.41	5.40		
	Triazole alanine	0.04	106.45	5.94	99.02	12.29
		1.0	91.59	13.61		



Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5	Overall recovery (%) n=10	RSD (%) n=10
	Triazole acetic acid	0.04	105.77	2.93	99.46	10.39
		1.0	93.16	12.29		
	Triazole-lactic-acid	0.04	104.72	1.87	100.20	8.66
		1.0	95.68	11.18		
Grape	1,2,4-Triazole	0.04	87.22	5.56	93.02	8.39
		1.0	98.82	5.48		
	Triazole alanine	0.04	99.34	5.90	103.67	5.85
		1.0	108.00	1.16		
	Triazole acetic acid	0.04	88.43	8.56	89.92	6.43
		1.0	91.42	3.84		
	Triazole lactic acid	0.04	82.31	5.50	89.24	9.20
		1.0	96.17	3.47		
Oilseed rape seeds	1,2,4-Triazole	0.04	91.42	8.77	95.35	7.71
		1.0	99.27	4.36		
	Triazole alanine	0.04	91.14	1.41	92.91	2.76
		1.0	94.68	2.42		
	Triazole acetic acid	0.04	91.12	5.93	93.92	5.96
		1.0	96.72	4.82		
	Triazole lactic acid	0.04	88.36	5.88	93.22	7.47
		1.0	98.08	4.89		
Peas dry seeds	1,2,4-Triazole	0.04	88.44	4.36	87.12	4.05
		1.0	85.79	3.46		
	Triazole alanine	0.04	82.29	4.02	86.60	7.48
		1.0	90.91	6.68		
	Triazole acetic acid	0.04	77.28	5.28	83.19	8.47
		1.0	89.09	3.19		
	Triazole lactic acid	0.04	74.25	2.86	84.75	13.82
		1.0	95.24	5.61		

**Table A 5: Recovery results from method validation of 1,2,4-Triazole, Triazole alanine, Triazole acetic acid and Triazole lactic acid using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (mg/kg) n = 5	Mean recovery (%) n=5	RSD (%) n=5
Apple	1,2,4-Triazole	0.04	101.28	2.23
	Triazole alanine	0.04	111.97	3.20
	Triazole acetic acid	0.04	108.79	6.84
	Triazole lactic acid	0.04	103.07	9.31
Grape	1,2,4-Triazole	0.04	76.99	4.68
	Triazole alanine	0.04	101.37	4.60
	Triazole acetic acid	0.04	84.75	3.14
	Triazole lactic acid	0.04	87.43	8.56
Oilseed rape seeds	1,2,4-Triazole	0.04	85.02	4.91
	Triazole alanine	0.04	97.26	2.45
	Triazole acetic acid	0.04	92.63	10.01
	Triazole lactic acid	0.04	91.67	12.55
Peas dry seeds	1,2,4-Triazole	0.04	85.74	5.46
	Triazole alanine	0.04	80.68	5.92
	Triazole acetic acid	0.04	75.11	3.42
	Triazole lactic acid	0.04	75.85	5.02

**Table A 6:** Characteristics of the analytical method used for the validation of 1,2,4-Triazole, Triazole alanine, Triazole acetic acid and Triazole lactic acid residues in apple, grape, oilseed rape seeds and peas dry seeds

	1,2,4-Triazole		Triazole alanine		Triazole acetic acid		Triazole lactic acid	
Specificity	MS spectra provided; blank value < 30 % LOQ for all matrices							
Calibration (matrix matched standard solutions at five concentration levels for all matrices)	apple							
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 139x – 525 R <sup>2</sup> > 0.99	y = 1000x – 762 R <sup>2</sup> > 0.99	y = 1101x – 928 R <sup>2</sup> > 0.99	y = 650x – 489 R <sup>2</sup> > 0.99	y = 2823x + 555 R <sup>2</sup> > 0.99	y = 190x + 191 R <sup>2</sup> > 0.99	y = 1208x + 2218 R <sup>2</sup> > 0.99	y = 210x – 340 R <sup>2</sup> > 0.99
	grape							
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 148x – 50 R <sup>2</sup> > 0.99	y = 1091x + 3403 R <sup>2</sup> > 0.99	y = 936x + 822 R <sup>2</sup> > 0.99	y = 484x – 205 R <sup>2</sup> > 0.99	y = 716x – 347 R <sup>2</sup> > 0.99	y = 45x + 61 R <sup>2</sup> > 0.99	y = 1039x + 5779 R <sup>2</sup> > 0.99	y = 192x + 776 R <sup>2</sup> > 0.99
	oilseed rape seeds							
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 258x – 19 R <sup>2</sup> > 0.99	y = 1900x + 662 R <sup>2</sup> > 0.99	y = 838x + 4975 R <sup>2</sup> > 0.99	y = 459x + 2455 R <sup>2</sup> > 0.99	y = 1753x – 420 R <sup>2</sup> > 0.99	y = 119x – 86 R <sup>2</sup> > 0.99	y = 1582x + 34 R <sup>2</sup> > 0.99	y = 270x + 175 R <sup>2</sup> > 0.99
peas dry seeds								

	1,2,4-Triazole		Triazole alanine		Triazole acetic acid		Triazole lactic acid	
	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)	calibration line equation (quantifier)	calibration line equation (qualifier)
	$y = 230x - 1$ $R^2 > 0.99$	$y = 1806x - 440$ $R^2 > 0.99$	$y = 1053x + 1587$ $R^2 > 0.99$	$y = 519x + 1009$ $R^2 > 0.99$	$y = 977x - 209$ $R^2 > 0.99$	$y = 66x - 16$ $R^2 > 0.99$	$y = 1354x - 994$ $R^2 > 0.99$	$y = 231x - 119$ $R^2 > 0.99$
Calibration range	For apple and grape from 0.006 µg/mL to 0.2 µg/mL, corresponding in matrix from 0.012 mg/kg to 0.4 mg/kg and 2 mg/kg (*); for and oilseed rape seeds and peas dry seeds from 0.0003 µg/mL to 0.1 µg/mL, corresponding in matrix from 0.012 mg/kg to 0.40 mg/kg and 2 mg/kg (*). (*): this data is obtained considering the maximum dilution factor applied to samples during analytical sequences (10 g of sample to 100 mL), done to include in the linearity range the recovery tests carried out at 1.0 mg/kg. Concentration ranges covered from 30% of the LOQ to 20% above the highest measured concentration							
Assessment of matrix effects is presented	yes							
Limit of quantification (LOQ)	0.04 mg/kg (for all matrices)							
Limit of detection (LOD)	0.012 mg/kg (for all matrices)							

## Conclusion

The analytical method was suitable to determine residues of 1,2,4-Triazole, Triazole alanine, Triazole acetic acid and Triazole lactic-acid in apple, grape, oilseed rape seeds and peas dry seeds. All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied for both primary and confirmatory detection.

Limit of quantification (LOQ) was successfully assessed to 0.04 mg/kg in all matrices and for all analyzed compounds.

### A 2.1.1.1.3 Analytical method 3

#### A 2.1.1.1.3.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/03

Report Validation of an HPLC-MS/MS analytical method for the determination of Prothioconazole and Azoxystrobin in water and sugar feeding solutions coming from ecotox laboratory tests (honeybees)  
Aversa S., 2020  
BT214/20

Guidelines: SANCO/3029/99 rev. 4 (11/07/2000)  
SANCO/825/00 rev.8.1 (16/11/2010)

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method adopted for the determination of Prothioconazole in water and sugar feeding solutions coming from ecotox laboratory tests is based on HPLC-MS/MS technique and has been validated following validity criteria required in SANCO/3029/99 rev.4 (11/07/2000) guidance document.

### Apparatus description

<i>HPLC</i>	Agilent UHPLC 1290 series with 6495a Triple Quadrupole Spectrometer
<i>Column</i>	Agilent Polaris 3 C8-ether 250 x 2.0 mm
<i>Eluent A</i>	0.1 % formic acid in water
<i>Eluent B</i>	acetonitrile
<i>Ratio A/B</i>	55/45
<i>Injection volume</i>	1 µL
<i>Column flow rate</i>	0.600 mL/min
<i>Column temperature</i>	35°C
<i>Run time</i>	4.5 minutes
<i>Acquiring mode</i>	ESI – positive
<i>Retention time</i>	Prothioconazole ≈ 2.6 min

Analyte	Identification	Precursor Ion	Product Ion	CE	Dwell	Cell Accel Voltage
Prothioconazole	Q1	344.04	154	40	200	2
	Q2	344.04	189	20	200	2

#### Validation data

The analytical method was validated in terms of Accuracy, Precision, Linearity, Selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANCO/825/00 rev. 8.1 (16/11/2010) and SANCO/3029/99, rev. 4 (11/07/2000) by means of recovery test and analysis of blank samples. Untreated samples were used for recovery test.

The following samples were analysed:

Matrix	Samples	Fortification level	Replicates in primary detection	Replicates confirmatory detection
water	control	-	2	2
	fortified	10 µg/L	5	5
	fortified	1000 µg/L	5	-
sugar feeding solutions	control	-	2	2
	fortified	1.0 mg/kg	5	5
	fortified	680 mg/kg	5	-

#### **Results and discussions**

In the following tables, a summary of validation data obtained in primary and confirmatory detection is reported.

#### Accuracy (as recovery) and precision (as repeatability)

A good accuracy was observed for all the fortified specimens analysed. All the recoveries were within the acceptable range of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was well below 20%.

**Table A 7: Recovery results from method validation of prothioconazole using the analytical method in primary detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water	prothioconazole	10 µg/L	98.29	4.59	
		1000 µg/L	104.22	3.88	
sugar feeding solutions	prothioconazole	1.0 mg/kg	101.76	3.28	
		680 mg/kg	97.0	3.0	

**Table A 8: Recovery results from method validation of prothioconazole using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water	prothioconazole	10 µg/L	101.58	3.93	
sugar feeding solutions	prothioconazole	1.0 mg/kg	101.49	5.02	

**Table A 9: Characteristics for the analytical method used for validation of prothioconazole residues in water and sugar feeding solutions**

	prothioconazole	
Specificity	MS spectra provided; blank value < 30 % LOQ for all matrices	
Calibration in solvent (water for both matrices) at five concentration levels in duplicate	calibration line equation (quantifier)	calibration line equation (qualifier)
	y = 534.4056x – 689.6050 R <sup>2</sup> > 0.99	y = 553.7602x – 1053.786 R <sup>2</sup> > 0.99
Calibration range	from 5.1792 µg/L to 155.3760 µg/L	
Assessment of matrix effects is presented	yes	
Limit of quantification in water (LOQ)	10.0 µg/L	
Limit of detection in water (LOD)	5.1792 µg/L	
Limit of quantification in water (LOQ)	1.0 mg/kg	
Limit of detection in water (LOD)	5.1792 µg/L	

## Conclusion

The analytical method was suitable to determine prothioconazole residues in water and sugar feeding solutions.

All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied for both primary and confirmatory detection.

Limit of quantification (LOQ) was successfully assessed to 10.0 µg/L in water and 1.0 mg/kg in sugar feeding solutions.

#### A 2.1.1.1.4 Analytical method 4

##### A 2.1.1.1.4.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.1.2/04

Report Validation of the analytical method (SANTE/2020/12830 Rev.1) for the determination of Prothioconazole and Prothioconazole-desthio in aqueous matrix and sugar feeding solutions with product SIP 41061  
Fifi A. P., 2022  
BT193/21

Guidelines: SANTE/2020/12830 rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

#### Materials and methods

The analytical method adopted for the determination of Prothioconazole in aqueous matrix and sugar feeding solutions with product SIP 41061 (Prothioconazole 400 g/L SC) and the analytical method for the determination of Prothioconazole-desthio in aqueous matrix solutions coming from the biological tests is based on HPLC-MS/MS technique and has been validated following validity criteria required in SANTE/2020/12830 rev.1 guidance document.

Sugar feeding solutions have been extracted weighting 1 g in a centrifuge tube. 1 mL of ultrapure water was added and then 10 mL of acetonitrile were added. The mixture was mixed for 1 minute and after mixing one spatula of sodium salt was added. The mixture was mixed again for 1 minute and then centrifuged at 5000 rpm for 5 minutes. The supernatant was opportunely diluted before the analysis.

#### Apparatus description (prothioconazole)

<i>HPLC</i>	Agilent UHPLC 1290 series with 6495a Triple Quadrupole Spectrometer					
<i>Column</i>	Phenomenex Kinetex C18 100Å, 2.6 µm, 3x50 mm					
<i>Eluent A</i>	0.1 % formic acid in water					
<i>Eluent B</i>	acetonitrile					
<i>Ratio A/B</i>	55/45					
<i>Injection volume</i>	1 µL					
<i>Column flow rate</i>	0.600 mL/min					
<i>Column temperature</i>	35°C					
<i>Run time</i>	4.5 minutes					
<i>Acquiring mode</i>	ESI – positive					
<i>Retention time</i>	Prothioconazole ≈ 2.5 min					

Analyte	Identification	Precursor Ion	Product Ion	CE	Dwell	Cell Accel Voltage
Prothioconazole	Quantifier	344.04	189	20	200	2
	Qualifier	344.04	154	40	200	2



#### Apparatus description (prothioconazole-desthio)

<i>HPLC</i>	Agilent UHPLC 1290 series with 6495a Triple Quadrupole Spectrometer
<i>Column</i>	Phenomenex Kinetex C18 100Å, 2.6 µm, 3x50 mm
<i>Eluent A</i>	0.1 % formic acid in water
<i>Eluent B</i>	acetonitrile
<i>Ratio A/B</i>	55/45
<i>Injection volume</i>	1 µL
<i>Column flow rate</i>	0.600 mL/min
<i>Column temperature</i>	35°C
<i>Run time</i>	4.5 minutes
<i>Acquiring mode</i>	ESI – positive
<i>Retention time</i>	Prothioconazole-desthio ≈ 1.7 min

Analyte	Identification	Precursor Ion	Product Ion	CE	Dwell	Cell Accel Voltage
Prothioconazole-desthio	Qualifier	312.1	125	40	200	3
	Quantifier	312.1	70	20	200	3

#### Validation data

The analytical method was validated in terms of accuracy, Precision, Linearity, Selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANTE/2020/12830 rev.1 by means of recovery test and analysis of blank samples.  
Untreated samples were used for recovery test.

The following samples were analysed:

Matrix	Analyte	Samples	Fortification level	Replicates in primary detection	Replicates confirmatory detection
water	prothioconazole	control	-	2	2
		fortified	0.0890 mg/L	5	5
		fortified	727.4150 mg/L	5	-
		fortified	124.7441 g/L	5	-
sugar feeding solutions	prothioconazole	control	-	2	2
		fortified	1.7533 g/Kg	5	5
		fortified	5.2673 g/Kg	5	-
water	prothioconazole-desthio	control	-	2	2
		fortified	0.1087 µg/L	5	5
		fortified	1.0873 µg/L	5	-

#### **Results and discussions**

In the following tables, a summary of validation data obtained in primary and confirmatory detection is reported.

**Table A 10: Recovery results from method validation of prothioconazole using the analytical method in primary detection**

Matrix	Analyte	Fortification level ( <i>n</i> = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water (M4 medium)	prothioconazole	0.0890 mg/L	90.54	3.55	
		727.4150 mg/L	89.21	2.66	
		124.7441 g/L	105.93	2.12	
sugar feeding solutions	prothioconazole	1.7533 g/Kg	96.0	3.38	
		5.2673 g/Kg	97.58	0.66	

**Table A 11: Recovery results from method validation of prothioconazole using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water (M4 medium)	prothioconazole	0.0890 mg/L	90.52	5.92	
sugar feeding solutions	prothioconazole	1.7533 g/Kg	94.27	1.87	

**Table A 12: Recovery results from method validation of prothioconazole-desthio using the analytical method in primary detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water (M4 medium)	prothioconazole – desthio	0.1087 µg/L	99.62	4.59	
		1.0873 µg/L	103.54	3.88	

**Table A 13: Recovery results from method validation of prothioconazole-desthio using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%) n=5	RSD (%) n=5	Comments
water (M4 medium)	prothioconazole – desthio	0.1087 µg/L	105.58	4.01	

**Table A 14: Characteristics for the analytical method used for validation of prothioconazole residues in aqueous matrix (M4 medium) and sugar feeding solutions**

Specificity	MS spectra provided blank value < 30 % LOQ for all matrices			
	M4 medium		sugar feeding solutions	
Calibration in matrix matched standard solutions at five concentration levels in duplicate	calibration curve equation (quantifier)	calibration line equation (qualifier)	calibration curve equation (quantifier)	calibration line equation (qualifier)
	$y = 1.039x^2 + 561.648x - 685.210$ $R^2 > 0.99$	$y = 1.048x^2 + 545.932x - 626.740$ $R^2 > 0.99$	$y = 0.279x^2 + 246.110x + 102.927$ $R^2 > 0.99$	$y = 0.233x^2 + 242.872x + 59.309$ $R^2 > 0.99$
Calibration range	from 3.0567 µg/L to 152.8365 µg/L (corresponding calibration range: 0.0306 mg/L – 1091.6893 mg/L)		from 3.0537 µg/L to 152.6865 µg/L (corresponding calibration range: 0.5090 g/kg – 7.6343 g/kg)	
Assessment of matrix effects is presented	yes			
Limit of quantification (LOQ)	0.0890 mg/L			
Limit of detection (LOD)	3.0567 µg/L			

**Table A 15: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in aqueous matrix (M4 medium)**

Specificity	MS spectra provided blank value < 30 % LOQ for all matrices	
Calibration in matrix matched standard solutions at five concentration levels in duplicate	calibration curve equation (quantifier)	calibration curve equation (qualifier)
	$y = 37710.592x^2 + 180650.245x + 9513.844$ $R^2 > 0.99$	$y = 12627.765x^2 + 61080.176x + 3341.137303$ $R^2 > 0.99$
Calibration range	from 0.0326 µg/L to 1.6310 µg/L (corresponding calibration range: 0.0326 µg/L – 1.6310 µg/L)	
Assessment of matrix effects is presented	yes	
Limit of quantification (LOQ)	0.1087 µg/L	
Limit of detection (LOD)	0.0326 µg/L	

## Conclusion

The analytical method was suitable to determine prothioconazole residues in water and sugar feeding solutions and prothioconazole-desthio residues in aqueous matrix.

All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied for both primary and confirmatory detection.

Limit of quantification (LOQ) was successfully assessed to 0.0890 mg/L in water and to 1.7533 g/Kg in sugar feeding solutions for prothioconazole and to 0.1087 µg/L in water for prothioconazole-desthio.

## A 2.1.1.1.5 Analytical method 5

### A 2.1.1.1.5.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/05
Report	Validation of an HPLC-MS/MS analytical method for the determination of Prothioconazole in soil coming from ecotox laboratory tests (earthworms) Aversa S., 2020 BT215/20
Guidelines:	SANCO/3029/99 rev. 4 (11/07/2000) SANCO/825/00 rev.8.1 (16/11/2010)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Materials and methods

The HPLC-MS/MS analytical method for the determination of Prothioconazole in soil was validated according to SANCO/3029/99 rev.4 (11/07/2000) guidance document.

About 10 g of dry soil were weighed in a 50 mL extraction tube. Then, 10 mL of acetonitrile and about 1 g of sodium chloride were added, the tube was hand shaken vigorously for 2 minutes. Finally, the tube was centrifuged at 5000 RPM for 5 minutes.

The obtained supernatant was filtered with 0.2 µm nylon filter and diluted, if necessary, in order to respect the linearity range. The obtained samples were finally analysed with a HPLC system coupled with a triple quadrupole mass analyser (LC-MS/MS).

### Apparatus description

<i>HPLC</i>	Agilent HPLC 1290 series with 6495b Triple Quadrupole Spectrometer
<i>Column</i>	Phenomenex Kinetex 2.6 µm C18 100Å 50 x 3 mm
<i>Eluent A</i>	Water + 0.1% formic acid
<i>Eluent B</i>	Acetonitrile
<i>Ratio A/B</i>	55/45
<i>Injection volume</i>	1.0 µL
<i>Column flow rate</i>	0.60 mL/min
<i>Column temperature</i>	35°C
<i>Run time</i>	4.5 minutes
<i>Acquiring mode</i>	ESI – positive
<i>Retention time</i>	Prothioconazole ≈ 2.5 min

Analyte	Identification	Precursor Ion	Product Ion	CE (V)	Dwell	Cell Accel Voltage
Prothioconazole-desthio	Q1 (quantifier)	344.04	189	20	200	2
	Qualifier	344.04	154	40	200	2

#### Validation data

The analytical method was validated in terms of accuracy, precision, linearity, selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANCO/825/00 rev. 8.1 (16/11/2010) and SANCO/3029/99, rev. 4 (11/07/2000) by means of recovery test and analysis of blank samples. Untreated samples were used for recovery test.

The following samples were extracted and analysed:

Samples	Fortification level	Replicates (in primary and confirmatory detection)
control	-	2
fortified	50 µg/Kg	5
fortified	430 mg/Kg	5

#### Results and discussions

In the following tables, a summary of validation data obtained in primary and confirmatory detection is reported.

#### Accuracy (as recovery) and precision (as repeatability)

A good accuracy was observed for all the fortified specimens analysed. All the recoveries were within the acceptable range of 70-110%. The repeatability, estimated as Relative Standard Deviation (RSD) was well below 20%.

**Table A 16: Recovery results from method validation of prothioconazole using the analytical method in primary detection**

Matrix	Analyte	Fortification level (n = 5)	Mean recovery (%)	RSD (%)	Comments
soil	prothioconazole	50 µg/Kg	96.08	0.45	
		430 mg/Kg	100.98	1.23	

**Table A 17: Recovery results from method validation of prothioconazole using the analytical method in confirmatory**

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
soil	prothioconazole	50 µg/Kg	96.43	1.07	

**Table A 18: Characteristics for the analytical method used for validation of prothioconazole residues in soil**

Specificity	MS spectra provided blank value < 30 % LOQ for all matrices	
Calibration in solvent at five concentration levels in double	calibration line equation (quantifier)	calibration line equation (qualifier)
	$y = 1275.443x - 7438.954$ $R^2 > 0.99$	$y = 1060.786x - 6228.879$ $R^2 > 0.99$
Calibration range	from 25.1142 µg/L to 125.5708 µg/L (corresponding to 0.0251 mg/Kg – 558.0919 mg/Kg)	
Assessment of matrix effects is presented	yes	
Limit of quantification (LOQ)	50 µg/Kg	
Limit of detection (LOD)	25.1142 µg/L	

## Conclusion

The analytical method was suitable to determine residues of prothioconazole in soil. All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied for both primary and confirmatory detection. Limit of quantification (LOQ) was successfully assessed to 50 µg/Kg for prothioconazole in soil.

### A 2.1.1.1.6 Analytical method 6

#### A 2.1.1.1.6.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/06
Report	Determination of dislodgeable foliar residue of prothioconazole and prothioconazole-desthio in raw agricultural commodity peach following two applications of SIP41061 (Prothioconazole 400 g/L SC) Desiante A., 2021 BIU-011-21
Guidelines:	SANTE/2020/12830, rev.1 (24/02/2021)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Materials and methods

The present analytical method has been developed to determine residues of Prothioconazole and Prothioconazole-desthio in the dislodging solution, obtained by the dislodging process, and validated under GLP compliance according to SANTE/2020/12830, rev.1 (24/02/2021) guideline.

The leaf discs were dislodged two times by 100 mL of the washing solution of 0.01% of dioctyl sulfosuccinate sodium salt (w/v). After each washing step, the solution was decanted and then transferred into a clean container adequately labelled. After the washing procedure, the leaf discs were discarded. Collection jar containing washing solution (200 mL approx.) was manually shaken and split in two aliquot of 100 mL approx.: one is the ship sample, one the retain sample.

The jar was stoppered and double bagged before freezing at -18°C.

The control leaf discs harvested for field fortification process were dislodged in the same way as all other specimens to obtain 200 mL of washing solutions each. Fortification was carried out on washing solution obtained.

The fortification was carried out after the arrival of the leaf disc specimens and just after the dislodging process.

After the fortification the sample obtained were split in ship and retain.

After fortification, the fortified specimens were retained in deep frozen condition to avoid any degradation.

A 5 mL portion of homogeneous sample was weighed into 50 mL centrifuge tube with screw capped, 5 ml of acetonitrile was added and shaken by vigorous manual stirring for 2 minutes.

The content of a QuEChERS Extraction kit was added and then shaken by vigorous manual stirring for 2 minutes. The sample was then centrifuged at 5000 RPM for about 5 minutes.

An aliquot of 1.5 mL of the extract was transferred into a vial and it's ready for the analysis.

#### Apparatus description

<i>HPLC</i>	Agilent 1290 Infinity liquid chromatograph / mass spectrometer mod. 6490 Agilent Triple Quadrupole with autosampler (LM2)				
	Agilent 1290 Infinity II liquid chromatograph / mass spectrometer Q-Trap 5500 Triple Quadrupole – ABSciex (LM3)				
<i>Column</i>	Synergi 2.5 µm MAX-RP 100Å 30x 2.0 mm - Phenomenex				
<i>Elution gradient</i>		<b>minutes</b>	<b>A%</b>	<b>B%</b>	<b>Flow (mL/min)</b>
		0.0	90	10	0.2
		2.0	40	60	
		4.0	40	60	
		5.0	90	10	
		7.0	90	10	
<i>Injection volume</i>	2.0 µL				
<i>Column flow rate</i>	0.20 mL/min				
<i>Column temperature</i>	30°C				
<i>Run time</i>	7.0 minutes				
<i>Acquiring mode</i>	ESI – positive				
<i>Retention time</i>	prothioconazole ≈ 3.6 min prothioconazole-desthio ≈ 3.3 min				

Analyte	Parent m/z	Product m/z	En. Collision (V)	Dwell	Transition name	Attribute
Prothioconazole	344	326	20	50	Prothio -1	Quantifier
		125	40	50	Prothio -2	Qualifier
Prothioconazole – desthio	312	70	35	50	M04 - 1	Quantifier
		125	35	50	M04 - 2	Qualifier

#### Validation data

The analytical method was validated in terms of Accuracy, Precision, Linearity, Selectivity, Limit of Quantification and Limit of Detection in compliance with guideline SANCO/3029/99, rev. 4 (11/07/2000) by means of recovery test and analysis of blank samples. Untreated samples were used for recovery test. The following samples were analysed:

Samples	Fortification level (µg/mL)	Replicates
control	-	2
fortified	0.01	7
fortified	0.1	7
fortified	1.0	7

#### **Results and discussions**

In the following tables, a summary of validation data is reported.

**Table A 19: Recovery results from method validation of prothioconazole and prothioconazole – desthio using the analytical method in primary detection**

Matrix	Analyte	Fortification level (mg/L) (n = 7)	Mean recovery (%)	RSD (%)	Comments
Dislodging foliar solution	prothioconazole	0.01	72.95	3.62	
		0.1	105.17	5.29	
		1.0	96.26	4.38	
	Prothioconazole – desthio	0.01	105.93	2.56	
		0.1	94.67	1.29	
		1.0	98.91	3.37	

**Table A 20: Recovery results from method validation of prothioconazole and prothioconazole – desthio using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (mg/L) (n = 7)	Mean recovery (%)	RSD (%)	Comments
Dislodging foliar solution	prothioconazole	0.01	76.25	3.80	
		0.1	105.43	5.30	
		1.0	97.63	4.23	
	prothioconazole – desthio	0.01	104.62	2.24	
		0.1	97.44	1.16	
		1.0	98.44	98.44	



**Table A 21: Characteristics for the analytical method used for validation of prothioconazole in foliar solutions coming from dislodging tests**

	prothioconazole		prothioconazole – desthio	
Specificity	MS spectra provided blank value < 30 % LOQ for all matrices			
Calibration (standard solutions at five concentration levels)	calibration curve equation (quantifier)	calibration curve equation (qualifier)	calibration curve equation (quantifier)	calibration curve equation (qualifier)
	y = 164x + 388  R <sup>2</sup> > 0.99	y = 625x – 396  R <sup>2</sup> > 0.99	y = 48945x + 41482  R <sup>2</sup> > 0.99	y = 17691x + 30609  R <sup>2</sup> > 0.99
Calibration range	from 0.003 µg/mL to 0.2 µg/mL			
Assessment of matrix effects is presented	yes			
Limit of quantification (LOQ)	0.01 mg/kg			
Limit of detection (LOD)	0.003 mg/kg			

## Conclusion

The analytical method was suitable to determine concentrations of prothioconazole and prothioconazole – desthio in solutions coming from dislodging tests.

All the requirements concerning specificity, recovery (accuracy), RSD (precision), limit of quantification and linearity were satisfied.

Limit of quantification (LOQ) was successfully assessed to 0.01 mg/kg.

### A 2.1.1.1.7 Analytical method 7

#### A 2.1.1.1.7.1 Method validation

Comments of zRMS:	Method is acceptable
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Reference: KCP 5.1.2/18

Report Prothioconazole-desthio and Hydroxy Metabolites: Method Validation in Honey  
Hitchens J.  
QG/21/009

Guidelines: SANTE/2020/12830, rev.1 (24/02/2021)

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The objective of this study was to validate an analytical method for the determination of residues of prothioconazole-desthio and (5) hydroxy metabolites in honey according to OECD, ENV/JM/MONO(2007)17 and SANTE/2020/12830 rev. 1.

Two methods of analysis were developed for prothioconazole-desthio and its 5 hydroxy metabolites.

Residues of prothioconazole-desthio were extracted from honey by dissolving the samples in water and then extracting the analyte with acetonitrile using QuEChERS kits. A portion of the final extract was then taken through the SPE clean-up and then diluted with water 2:3 v/v for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Two ion mass transitions were monitored, except for stability samples where the quantitation transition only was monitored. The method was validated in terms of linearity (calibration), specificity (selectivity), accuracy (recovery), precision (repeatability), matrix effects and stability of extracts and standards.

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

## Apparatus description for prothioconazole-desthio determination

Column	Kinetex 5 μm XB-C18 100A, 150 x 4.6 mm				
Guard column	C18 4 x 3.0 mm – Part no. AJ0-4287				
MS/MS system	API 5000				
Mobile Phase A:	0.1 % formic acid in water				
Mobile Phase B:	0.1 % formic acid in methanol				
Elution gradient		minutes	A %	B %	Flow (μL/min)
		0.00	40	60	1000
		1.00	40	60	
		5.00	10	90	
		6.00	10	90	
		6.10	0	100	
		7.00	0	100	
		7.10	40	60	
		9.00	40	60	
Injection volume	60 μL				
Column flow rate	0.20 mL/min				
Column temperature	40°C				
Run time	6.0 minutes				
Acquiring mode	ESI – positive				
Retention time	5.3 min				

Analyte	Q1 mass	Q3 mass	En. Collision (V)	Dwell time (msec)	Transition name
Prothioconazole – desthio	312.1	70.1	20	250	1#
		125.0	30	250	2#

### Apparatus description for prothioconazole-hydroxy-desthio metabolites determination

Column	Kromasil 5 μm C18 100A 4.6 × 250 mm					
Guard column	C18 4 x 3.0 mm – Part no. AJ0-4287					
MS/MS system	API 5500					
Mobile Phase A:	0.1 % formic acid in water					
Mobile Phase B:	methanol					
Elution gradient		minutes	A %	B%	Flow (μL/min)	
		0.00	40	60	1000	
		1.00	40	60		
		5.00	10	90		
		6.00	10	90		
		6.10	0	100		
		7.00	0	100		
		7.10	40	60		
		9.00	40	60		
Injection volume	20 μL					
Column flow rate	0.20 mL/min					
Column temperature	40°C					
Run time	14.9 minutes					
Acquiring mode	ESI – positive					
Retention time	Prothioconazole-α-hydroxy-desthio ≈ 6.06 and 8.2 min Prothioconazole-3-hydroxy-desthio ≈ 7.7 min Prothioconazole-4-hydroxy-desthio ≈ 8.8 min Prothioconazole-5-hydroxy-desthio ≈ 9.3 min Prothioconazole-6-hydroxy-desthio ≈ 14.3 min					

Analyte	Q1 mass	Q3 mass	En. Collision (V)	Dwell time (msec)	Transition name
Prothioconazole-α-hydroxy-desthio	328.265	69.9	36	100	1#
		141.2	39	100	2#
Prothioconazole-3-hydroxy-desthio	328.142	70.0	37	100	1#
		141.0	47	100	2#
Prothioconazole-4-hydroxy-desthio	328.154	70.0	35	100	1#
		141.0	53	100	2#
Prothioconazole-5-hydroxy-desthio	327.948	70.0	33	100	1#
		141.0	45	100	2#
Prothioconazole-6-hydroxy-desthio	327.899	140.872	68	100	1#
		69.824	44	100	2#

### Validation data

Two ion mass transitions were monitored, except for stability samples where the quantitation transition only was monitored. The method was validated in terms of linearity (calibration), specificity (selectivity), accuracy (recovery), precision (repeatability), matrix effects and stability of extracts and standards in compliance with guideline SANTE/2020/12830 rev. 1.

The following samples were analysed:

Samples	Fortification level (µg/mL)	Replicates
control	-	2
fortified	0.005	5
fortified	0.05	5

## Results and discussions

In the following tables, a summary of validation data is reported.

**Table A 22: Recovery results from method validation of prothioconazole-desthio and prothioconazole-hydroxy metabolites in honey using the analytical method in primary detection**

Matrix	Analyte	Fortification level (mg/L) (n = 7)	Mean recovery (%)	RSD (%)	Comments
Honey	prothioconazole – desthio	0.005	106	1.6	
		0.05	107	2.1	
	prothioconazole – α-hydroxy-desthio	0.005	85	1.6	
		0.05	84	10	
	prothioconazole – 3-hydroxy-desthio	0.005	89	0.6	
		0.05	86	9.5	
	prothioconazole – 4-hydroxy-desthio	0.005	80	1.6	
		0.05	83	2.9	
	prothioconazole – 5-hydroxy-desthio	0.005	88	1.4	
		0.05	87	10	
	prothioconazole – 6-hydroxy-desthio	0.005	88	9.7	
		0.05	78	2.4	

**Table A 23: Recovery results from method validation of prothioconazole-desthio and prothioconazole-hydroxy metabolites in honey using the analytical method in confirmatory detection**

Matrix	Analyte	Fortification level (mg/L) (n = 7)	Mean recovery (%)	RSD (%)	Comments
Honey	prothioconazole – desthio	0.005	106	1.0	
		0.05	105	2.0	
	prothioconazole – $\alpha$ -hydroxy-desthio	0.005	86	4.0	
		0.05	87	2.3	
	prothioconazole – 3-hydroxy-desthio	0.005	88	1.7	
		0.05	86	8.4	
	prothioconazole – 4-hydroxy-desthio	0.005	80	1.1	
		0.05	83	2.5	
	prothioconazole – 5-hydroxy-desthio	0.005	87	1.6	
		0.05	90	2.8	
	prothioconazole – 6-hydroxy-desthio	0.005	89	10.9	
		0.05	78	2.8	

**Table A 24: Characteristics for the analytical method used for validation of prothioconazole-desthio and prothioconazole- $\alpha$ -hydroxy-desthio in honey**

	prothioconazole-desthio		prothioconazole- $\alpha$ -hydroxy-desthio	
Specificity	MS spectra provided blank value < 30 % LOQ for all matrices			
Calibration (standard solutions at five concentration levels)	calibration curve equation (quantifier)	calibration curve equation (qualifier)	calibration curve equation (quantifier)	calibration curve equation (qualifier)
	y = 1.22e+5x + 1.14e+3	y = 1.49e+5x + 203	y = 2.51x + 4.53e+3	y = 5.72x + 1.58e+3
	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99
Calibration range	from 0.05 ng/mL to 2.5 ng/mL, corresponding to an equivalent sample concentration of 0.00125 to 0.0625 mg/kg		from 0.375 ng/mL to 15.0 ng/mL, corresponding to an equivalent sample concentration of 0.0015 to 0.060 mg/kg	
Assessment of matrix effects is presented	yes			
Limit of quantification (LOQ)	0.005 mg/kg		0.005 mg/kg	
Limit of detection (LOD)	0.00125 mg/kg		0.0015 mg/kg	

**Table A 25: Characteristics for the analytical method used for validation of prothioconazole-3-hydroxy-desthio and prothioconazole-4-hydroxy-desthio in honey**

	prothioconazole-3-hydroxy-desthio		prothioconazole-4-hydroxy-desthio	
Specificity	MS spectra provided blank value < 30 % LOQ for all matrices			
Calibration (standard solutions at five concentration levels)	calibration curve equation (quantifier)	calibration curve equation (qualifier)	calibration curve equation (quantifier)	calibration curve equation (qualifier)
	y = 2.24e+5x + 556	y = 1.15e+5x + 2.69e+3	y = 2.31x + 4.18e+3	y = 1.91e+5x + 7.61e+3
	R² > 0.99	R² > 0.99	R² > 0.99	R² > 0.99
Calibration range	from 0.375 ng/mL to 15.0 ng/mL, corresponding to an equivalent sample concentration of 0.0015 to 0.060 mg/kg		from 0.375 ng/mL to 15.0 ng/mL, corresponding to an equivalent sample concentration of 0.0015 to 0.060 mg/kg	
Assessment of matrix effects is presented	yes			
Limit of quantification (LOQ)	0.005 mg/kg		0.005 mg/kg	
Limit of detection (LOD)	0.00125 mg/kg		0.0015 mg/kg	

**Table A 26: Characteristics for the analytical method used for validation of prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in honey**

	prothioconazole-5-hydroxy-desthio		prothioconazole-6-hydroxy-desthio	
Specificity	MS spectra provided blank value < 30 % LOQ for all matrices			
Calibration (standard solutions at five concentration levels)	calibration curve equation (quantifier)	calibration curve equation (qualifier)	calibration curve equation (quantifier)	calibration curve equation (qualifier)
	y = 1.71e+5x + 1.78e+3	y = 9.05e+4x + 3.75e+3	y = 5.03e+4x + 3.65e+3	y = 2.98e+5x + 5.08e+3
	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99	R <sup>2</sup> > 0.99
Calibration range	from 0.375 ng/mL to 15.0 ng/mL, corresponding to an equivalent sample concentration of 0.0015 to 0.060 mg/kg		from 0.375 ng/mL to 15.0 ng/mL, corresponding to an equivalent sample concentration of 0.0015 to 0.060 mg/kg	
Assessment of matrix effects is presented	yes			
Limit of quantification (LOQ)	0.005 mg/kg		0.005 mg/kg	
Limit of detection (LOD)	0.00125 mg/kg		0.0015 mg/kg	

## Conclusion

The analytical method was successfully validated in accordance with the requirements of SANTE/2020/12830 rev.1, and is considered suitable for the determination of residues of

prothioconazole-desthio and prothioconazole-desthio (5) hydroxy metabolites in honey with an LOQ of 0.005 mg/kg for each analyte

**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**

No new or additional studies have been submitted