FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: Protiokonazol 300 EC

Product name(s): HERA 300 EC

Chemical active substance:

prothioconazole, 300 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: Pestila Spółka z ograniczoną odpowiedzialnością

Submission date: October 2023

MS Finalisation date: April 2024; July 2024

Version history

When	What
April 2024	ZRMs evaluated dRR submitted by Applicant.
July 2024	The final Registration Report

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5 Analytical methods

5.1 Conclusion and summary of assessment

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

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

Commodity/crop	Supported/ Not supported
Winter wheat	Supported
Spring wheat	Supported
Winter triticale	Supported
Spring triticale	Supported
Spring barley	Supported
Winter barley	Supported
Rye	Supported
Winter oilseed rape	Supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of zRMS:	The proposed analytical method is suitable for the determination of active substance prothioconazole in plant protection product Protiokonazol 300 EC (Hera 300 EC). The proposed analytical method has been fully validated in terms of specificity, linearity, repeatability, and accuracy. The proposed method fulfils the requirements of SANCO/3030/99 rev.5 guidance. The validation of the analytical method has been accepted.
Reference:	5.1.1/01
Report	Protiokonazol 300 EC. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage, Łysik A., 2022, report no. BF – 25/22
Guideline(s):	Yes, SANCO/3030/99 rev.5 (22/03/19)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Determination of prothioconazole in Protiokonazol 300 EC was performed with reversed phase high performance liquid chromatography (RP-HPLC) with UV-Vis detection at wavelength 206 nm and external standard.

Equipment and chromatographic conditions for prothioconazole analysis

- Shimadzu liquid chromatograph equipped with UV-Vis detector
- Column: Kinetex Biphenyl, 250x4.6mm, 5µm
- Analytical balance Mettler Toledo AT261, accuracy 0.01 mg
- Glass pipettes
- Volumetric flasks
- Autosampler vials
- Ultrasonic bath, POLSONIC
- Typical laboratory equipment
- Prothioconazole, 98.9%
- Analytical standards
- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC, POCh
- o-Phosphoric acid, CHEMPUR
- Oven temperature: 30ºC
- Flow rate: 1 ml/min
- Wavelength λ = 206 nm
- Injection volume: 5 µl

- Mobile phase composition: acetonitrile + 0.1 % H_3PO_4 (40+60) (v/v)

Under the above conditions the retention time for Prothioconazole is 25.5 min ± 0.3 min. Total time of analysis is 30 min.

The preparation of standard solution

8.24 mg of Prothioconazole standard was weighed (with the accuracy of 0.01 mg) into the 25 ml volumetric flask and acetonitrile was added to the nominal volume. Solution was diluted and analyzed.

The preparation of specimen solutions

About 10 mg of examined specimen was weighed (with the accuracy of 0.01 mg) into the 10 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume. Solution was diluted and analyzed.

The preparation of placebo solution

18.81 mg of placebo was weighed (with the accuracy of 0.0 mg) into the 25 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume. Solutions was diluted and analyzed.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances prothioconazole in plant protection product Protiokonazol 300 EC

	Prothioconazole
Author(s), year	Łysik A, 2022
Principle of method	SANCO/3030/99 rev.5, 22 March 2019
Linearity **n = 5** **(linear between** **mg/L / % range of the declared content)** **(correlation coefficient, expressed as r)**	The linearity of the analytical method was assessed using five prothioconazole standard solutions in the concentration range from 0.0196 mg/mL to 0.0489 mg/mL. Correlation coefficient: $R^2 = 0.994$ Required: $R^2 \geq 0.99$
Precision – Repeatability Mean **n = 6** **(%RSD)**	Hr = 0.48 Required: Hr ≤ 1 RSD = 0.78 Required: RSD ≤ 1.62
Accuracy **n = 12** **(% Recovery)**	Total recovery: 99.72% (range: 97.2% - 102.7%) Required: 97% - 103%
Interference/ Specificity	Fulfilled. Chromatograms of solvent, specimen of Protiokonazol 300 EC solution, placebo solution, and standard solution were performed and superimposed. There are no other peaks that could interfere with peak of prothioconazole under the specified chromatographic conditions.
Comment	No comments.

Conclusion

The RP-HPLC method, used to quantify prothioconazole in Protiokonazol 300 EC was fully validated. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

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

Comments of zRMS:	The analytical methods for the determination of relevant impurities (prothioconazole-desthio and toluene) in plant protection product Protiokonazol 300 EC (Hera 300 EC) are suitable for the determination of the content of each of the relevant impurity in the presence of each other, active substance and other components. The methods have been fully validated. The validation parameters of proposed analytical methods – interference, specificity, linearity, recovery, repeatability, and LOQ values fulfil the requirements of SANCO/3030/99 rev. 5 guidance. The validation of the analytical methods has been accepted.
Reference:	5.1.1/02
Report	Protiokonazol 300 EC. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage, Łysik A., 2022, report no. BF – 25/22
Guideline(s):	Yes, SANCO/3030/99 rev.5 (22/03/19)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods for Prothioconazole-desthio content

Determination of Prothioconazole-desthio in Protiokonazol 300 EC was performed with reversed phase high performance liquid chromatography (RP-HPLC) with UV-Vis detection at wavelength 206 nm and external standard.

Equipment and chromatographic conditions for Prothioconazole-desthio analysis

- Shimadzu liquid chromatograph equipped with UV-Vis detector
- Column: Luna Omega, 150x4.6mm, 5µm
- Analytical balance Mettler Toledo AT261, accuracy 0.01 mg
- Glass pipettes
- Volumetric flasks
- Autosampler vials
- Ultrasonic bath, POLSONIC
- Typical laboratory equipment
- Prothioconazole-desthio, 97.94%,
- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC, POCh
- o-Phosphoric acid, CHEMPUR
- Oven temperature: 40ºC
- Flow rate: 1 ml/min
- Wavelength λ = 206 nm
- Injection volume: 5 µl

- Mobile phase composition: acetonitrile + 0.1% H3PO4 (45 + 55) (v/v)
- Under the above conditions the retention time for Prothioconazole-desthio is 10.6 min ± 0.3 min. Total time of analysis is 40 min.
- Precursor ion m/z: 312.1 / 314.1
- Product ions m/z: 125.1 and 70.1 / 125.1 and 70.1

The preparation of standard solution

86.50 mg of Prothioconazole-desthio standard was weighed (with the accuracy of 0.01 mg) into the 25 ml volumetric flask and acetonitrile was added to the nominal volume. Solution was diluted and analyzed.

The preparation of specimen solutions

About 200 mg of examined specimen was weighed (with the accuracy of 0.01 mg) into the 10 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume and analyzed.

The preparation of placebo solution

2530.97 mg of placebo was weighed (with the accuracy of 0.0 mg) into the 25 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume. Solutions was diluted and analyzed.

Materials and methods for toluene content

The content of toluene in the examined preparation was determined using headspace analysis in combination with gas chromatography and flame ionization detection (HS-GC-FID) using external standard-toluene.

Equipment and chromatographic conditions for toluene analysis

- VARIAN CP-3800 Gas Chromatograph with FID
- Teledyne Tekmar HT-3 Headspace Autosampler
- Rxi®-1301Sil MS capillary column, 30 m × 0.25 mm × 1.0 μm (RESTEK)
- Analytical balance Mettler Toledo XS205 Dual Range, accuracy of 0.01 mg
- Glass pipettes
- Automatic pipettes
- Glass graduated flasks
- 20 mL headspace vials with alumina caps and Teflon-silicone septa
- Laboratory crimper
- Autosampler vials
- Typical laboratory equipment
- Toluene, 99.94%
- Dimethyl sulfoxide (DMSO), for headspace analysis, VWR Chemicals
- Chromatographic conditions
- Oven: 45 ºC (1 min), 5ºC/min → 80ºC, 30ºC /min → 250ºC (1 min)
- Carrier gas: Helium
- Flow: 2 mL/min
- Inlet temperature: 250ºC
- Detector temperature: 300ºC
- Split ratio: 1:20
- Auxiliary gases flow:
- nitrogen: 25 mL/min
- hydrogen: 30 mL/min
- air: 300 mL/min

- Valve oven temperature: 90ºC
- Transfer line temperature: 100ºC
- Platen/sample temperature: 60ºC
- Sample equilibration time: 10 min
- Mixing time: 3 min (Level 7)
- Pressurization: 6 psig
- Loop fill pressure: 5 psig
- Loop volume: 1 mL
- Injection time: 1 min

Under the above conditions retention time of toluene was 7.06 ± 0.05 min and the total time of analysis is 14.67 min. Mass spectra were performed using EI technique (70 eV) in full scan mode from 40 to 300 m/z.

The preparation of standard solution

About 50 mg of toluene standard were weighed (with the accuracy of 0.01 mg) into two separate 5 ml flasks and DMSO was added up to the volume. Solution was diluted and analyzed.

The preparation of specimen solutions

About 50 mg of the examined preparations were placed in four headspace vials and then 2 mL DMSO was added and analyzed.

The preparation of fortified placebo solution

In six headspace vials about 40 mg of placebo were placed. To three headspace vials 1.900 mL of DMSO and 0.100 mL of working standard solution B1 was added (0.010 mg of toluene) and to other three 1.800 mL of DMSO and 0.200 mL of working standard solution B2 was added (0.020 mg of toluene). The vials were tightly closed and then analyzed. Headspace of each of the above-mentioned solutions was analyzed.

Table 5.2-2: Methods suitable for the determination of the relevant impurities: Prothioconazole-desthio and toluene in plant protection product (PPP) Protiokonazol 300 EC

	Prothioconazole-desthio	**Toluene**
Author(s), year	Łysik A, 2022	
Principle of method	SANCO/3030/99 rev.5, 22 March 2019	
Linearity **n = 6** **(linear between mg/L)** **(correlation coefficient, expressed as r)**	The linearity of the analytical method was assessed using six standards solutions of Prothioconazole-desthio in the concentration range from 0.00030 mg/mL to 0.00392 mg/mL. Correlation coefficient: $R^2 = 0.9990$ Required: $R^2 \geq 0.99$.	The linearity of the analytical method was assessed using six standards solutions of Toluene in the concentration range from 0.002 to 0.0200 mg. Correlation coefficient: $R^2 = 0.9992$ Required: $R^2 \geq 0.99$.
Precision – Repeatability Mean **n = 5 (Prothioconazole-desthio)** **n = 6 (Toluene)** **(%RSD)**	Hr = 0.81 Required: Hr ≤ 1 RSD = 4.6 Required: RSD ≤ 5.69.	Hr = 0.41 Required: Hr ≤ 1 RSD = 1.93 Required: RSD ≤ 4.75
Accuracy **n = 12 (Prothioconazole-desthio)** **n = 6 (Toluene)** **(% Recovery)**	Total recovery: 101.3% (range: 79.3% - 119.5%) Required: 70% - 130%.	Total recovery: 101.9% (range: 97.7% - 106.8%) Required: 75% - 125%.

	Prothioconazole-desthio	Toluene
Interference/ Specificity	Fulfilled. Chromatograms of the solvent, standard, placebo, and Protiokonazol 300g/L EC (Hera 300 EC) sample solutions were performed and superimposed. There are no other peaks that could interfere with the Prothioconazole-desthio peak under the specified chromatographic conditions. MS/MS spectra were attached.	Fulfilled. To prove specificity of the developed method the following headspace chromatograms (HS-GC-FID) were performed and superimposed: DMSO (sample solvent, 2mL), placebo in DMSO (40 mg + 2 ml), specimen (Protiokonazol 300 EC in DMSO (50 mg+2 mL)) and standard solution of toluene in DMSO (0.010 mg in 2 mL). Chromatograms were attached.
LOQ	0.00015% (0.0015 g/kg of the preparation).	0.004% (0.04 g/kg) of the preparation.
Comment	No comments.	No comments.

Conclusion

Determination of ~~residues of sum of dioxins and furans~~ toluene was fully validated. The methods for determination are specific. The validation parameters for linearity, instrument precision, limit of quantification, repeatability and accuracy are within the acceptance range. There are not any interferences between relevant impurities and other ingredients of the samples. The methods had good precision, accuracy and the linearity and fulfil requirements of SANCO/3030/99 rev.5.

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

Not relevant. The product Protiokonazol 300 EC does not contain materials of toxicological, ecotoxicological or environmental concern.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For prothioconazole emulsifiable concentrate CIPAC Method 745 (CIPAC Handbook P, page 169) is suitable.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of prothioconazole for the generation of pre-authorization data is given in the following table.
Additionally, analytical methods for the determination of prothioconazole in the ecotoxicology and residue studies are provided with this application. The detailed evaluation of additional studies, it is referred to Appendix 2. New data has been generated for TDMs. Please refer to the data to which the applicant has been granted access under the LoA.

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

Component of residue definition: Prothioconazole: Prothioconazole-desthio (sum of isomers)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**

Component of residue definition: Prothioconazole: Prothioconazole-desthio (sum of isomers)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Honey (Residues)	Primary & confirmatory	0.01 mg/kg (Prothioconazole) 0.01 mg/kg (Prothioconazole-desthio)	HPLC-MS/MS + triple-quadrupole mass spectrometry, acquiring 2 simultaneous MRM transitions	Peda T., 2022 / Study code: 22SGS46; LBN-0044-2022
		0.01 mg/kg (TDM)	HPLC-MS/MS + triple quadrupole mass spectrometry, equipped with a differential mobility separation device (DMS) acquiring 2 simultaneous MRM transitions	
Soil (*Eisenia fetida*) (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1139.1F.SAG22, Test Site Code: 22293-02R
Water (*Apis mellifera* L. - Larval Toxicity Test) (Ecotoxicology)	Primary & confirmatory	0.00025 g/L	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1002.1F.SAG22; Test Site Code: 22293-03R
Soil (*Hypoaspis (Geolaelaps) aculeifer)* (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1142.1F.SAG22; Test Site Code: 22293-04R
Sucrose solutions and water (*Apis mellifera* L. - Chronic Oral Toxicity*)* (Ecotoxicology)	Primary & confirmatory	0.8576 mg/kg (in sucrose solution) 0.00025 g/L (in water)	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1001.1F.SAG22; Test Site Code: 22293-01R
Soil (*Folsomia candida)* (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1143.1F.SAG22; Test Site Code: 22293-05R
ISO standard water (*Daphnia magna)* (Ecotoxicology)	Primary & confirmatory	0.020 mg/L	HPLC - LC-MS/MS	Artusio M., 2023 / Study Code: 1136.1F.SAG22; Test Site Code: 22293-06R
Sucrose solutions and water (*Bombus terrestris* L. - Acute Oral and Contact Toxicity*)* (Ecotoxicology)	Primary & confirmatory	0.8576 mg/kg (in sucrose solution) 0.000245 g/L (in water)	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1138.1F.SAG22; Test Site Code: 22293-07R
Water (Non-target plants – Seedling Emergence and	Primary & confirmatory	0.00025 g/L	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: Study Code: 1140.1F.SAG22; Test Site Code: 22293-

Component of residue definition: Prothioconazole: Prothioconazole-desthio (sum of isomers)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Seedling growth) (Ecotoxicology)				08R
Water (Terrestrial Plant Vegetative Vigour) (Ecotoxicology)	Primary & confirmatory	0.00025 g/L	HPLC - LC-MS/MS	Mautino G., 2023 / Study Code: 1141.1F.SAG22; Test Site Code: 22293-09R
Aqueous solutions (*Raphidocelis subcapitata*) (Ecotoxicology)	Primary & confirmatory	7.6 µg/L	HPLC/MS-QQQ	Tediosi E., 2023 / Study Code: CH – 0912-2022

zRMS:
The applicant has been granted access under the LoA (Indofil Industries (Netherlands) B.V.) to following studies:

- Longhi, D., 2021c. Validation of an analytical method for the quantification of the Prothioconazole-desthio-3-hydroxy, Prothioconazole-desthio-4-hydroxy, Prothioconazole-desthio-5-hydroxy, Prothioconazole-desthio-6-hydroxy, Prothioconazole-desthio-alpha-hydroxy in cereal straw. Report No.: 21-120.

The analytical method was based on the QuEChERS method and the instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography-triple-quadrupole mass spectrometry). The validation of the analytical method was carried out under GLP compliance according to SANTE/2020/12830 rev.1 guideline. The method is acceptable.
LOQ=0.01 mg/kg.

- Longhi, D., 2021b. Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities Report No GLP-STUDY-21-108

Part of the study for which the access is granted: Validation of an analytical method for determination of TDM (TRZ, TA, TLA, TAA) in Whole Plant (Rapeseed), wheat grain, white bread, straw.
Method was accepted in the *Prothioconazole_fRR Part B5_INDOFIL Prothio 250 EC_Indofil Industries_PL_rev._02.2024.*
The validation of the analytical method was carried out under GLP compliance to SANTE/2020/12830 Rev.1 guideline. The analytical determination was carried out using a HPLC-MS/MS method. LOQ= 0.01 mg/kg (wheat grain and straw; white bread (wheat); beer (barley)) for 1,2,4-TRZ, TA, TLA and TAA.

- Longhi, D., 2021a. Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities Report No GLP-STUDY-21-31

Part of the study for which the access is granted: Validation of an analytical method for determination of Prothioconazole-desthio in Whole Plant (Rapeseed), wheat grain, white bread, straw.
Method was accepted in the *Prothioconazole_fRR Part B5_INDOFIL Prothio 250 EC_Indofil Industries_PL_rev._02.2024.*
The analytical method for the determination of Difenoconazole and Prothioconazole-desthio in the tested matrices was based on the QuEChERS method (EN 15662_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry). LOQ=0.01 mg/kg (wheat grain and straw; white bread (wheat); beer (barley)).

- Nichetti, S., 2022a. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat). Study No. CH-1081-2021

Part of the study for which the access is granted: ILV of the Analytical Method for the Determination of Prothio-desthio in Straw (wheat).
Method was accepted in the *Prothioconazole_fRR Part B5_INDOFIL Prothio 250 EC_Indofil Industries_PL_rev._02.2024.*
Analysis by HPLC using external standards and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode. LOQ=0.01 mg/kg.

- Nichetti, S. 2022b. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Grain (wheat). Study No. CH-1082-2021

Part of the study for which the access is granted: ILV of the Analytical Method for the Determination of Prothio-desthio in Grain (wheat).
Method was accepted in the *Prothioconazole_fRR Part B5_INDOFIL Prothio 250 EC_Indofil Industries_PL_rev._02.2024.*
Analysis by HPLC using external standards and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode. LOQ=0.01 mg/kg.

- Nichetti, S. 2022d. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole plant (rapeseed). Study No. CH-1084-2021

Part of the study for which the access is granted: ILV of the Analytical Method for the Determination of Prothio-desthio in Whole plant (rapeseed).
Method was accepted in the *Prothioconazole_fRR Part B5_INDOFIL Prothio 250 EC_Indofil Industries_PL_rev._02.2024.*

- Nichetti, S. 2022g. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed). Report No.: CH-1085/2021.
- Nichetti, S. 2022i. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Straw (wheat). Report No.: CH-1086/2021.
- Nichetti, S. 2022h. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Grain (wheat). Report No.: CH-1087/2021.
- Nichetti, S. 2022e. Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Cereal Straw. Report No.: CH-1091/2021.

Report No. CH-1085/2021, CH-1086/2021, CH-1087/2021, CH-1091/2021 and Longhi, D., 2021c have been submitted for registration application of Indofil's plant protection product, Avtar (Product code: IN233C1560) which is currently undergoing zonal evaluation (by the z-RMS Poland).

The applicant does not have access to a copies of the studies. Therefore, there are no study summaries in the documentation.

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substance in the plant protection product are already submitted in accordance with the requirements set out in point 5.2.1.

5.3.2 Description of analytical methods for the determination of residues nicosulfuron (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)	0.01 mg/kg (0.1 mg/kg for wheat incl. triticale, 0.2 mg/kg for barley, 0.05 mg/kg for rye)*	Reg. (EU) No. 2019/552
Plant, high acid content		0.01 mg/kg	
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	
Plant, high oil content		0.02 mg/kg (0.15 mg/kg for oilseed rape)*	
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg	
Muscle	Prothioconazole-desthio (sum of isomers)	0.01 mg/kg	Reg. (EU) No. 2019/552
Milk		0.01 mg/kg	
Eggs		0.01 mg/kg	
Fat		0.02 mg/kg	
Liver, kidney		0.5 mg/kg	
Soil (Ecotoxicology)	Prothioconazole and prothioconazole-desthio	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Prothioconazole and prothioconazole-desthio	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Prothioconazole and prothioconazole-desthio	0.308 mg a.s./L (prothioconazole) 3.34 µg/L (prothioconazole-desthio)	NOEC *Oncorhynchus mykiss* EFSA Journal 2007; 106, 1-98

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Air	Prothioconazole	60 µg/m^3 (prothioconazole) (AOEL sys = 0.2 mg/kg bw/day) 3 µg/m^3 (prothioconazole-desthio) (AOEL sys = 0.01 mg/kg bw/d)	Calculated according to SANTE/2020/12830, Rev.1 24. February 2021
Tissue (meat or liver)	-	not required	not classified as T / T+
Body fluids		not required	not classified as T / T+

* Post-authorisation methods comply with the currently in force MRL for wheat incl. triticale (0.1 mg/kg), barley (0.2 mg/kg), rye (0.05 mg/kg) and oilseed rape (0.15 mg/kg).

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in plant matrices is given in the following tables. New data has been generated for TDMs. Please refer to the data to which the applicant has been granted access under the LoA.

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 (sum of isomers)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary & confirmatory	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Weeren, R.D. Pelz, S. 2000, (DAR, 2005)
	ILV	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Class, T. 2001, (DAR, 2005)
High acid content	Primary & confirmatory	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Weeren, R.D. Pelz, S. 2000, (DAR, 2005)
	ILV	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Class, T. 2001, (DAR, 2005)
High oil content	Primary & confirmatory	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Weeren, R.D. Pelz, S. 2000, (DAR, 2005)
	ILV	LOQ 0.02 mg/kg	GC-MS (DFG S19)	Class, T. 2001, (DAR, 2005)
High protein/high starch content (dry)	Primary & confirmatory	LOQ 0.05 mg/kg	GC-MS (DFG S19)	Weeren, R.D. Pelz, S. 2000, (DAR, 2005)
	ILV	LOQ 0.05 mg/kg	GC-MS (DFG S19)	Class, T. 2001, (DAR, 2005)

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Weeren, Pelz 2000 (DFG S19), DAR, 2005
Not required, because:	-

zRMS comment: The LOQs of the EU agreed methods cited in the table do not meet the current legal MRLs in Reg (EU) No 2019/552.
The proposed GAP uses of Protiokonazol 300 EC are on cereal crops only, for which the analytical methods relied upon have sufficient LOQs to meet the current legal MRLs as listed in Reg (EU) No 2019/552. Under Article 33 of Regulation (EC) No 1107/2009, the current EU-agreed endpoints are relevant. Analytical methods data accepted in the 2004 DAR and 2007 DAR Addendum (RMS: UK), and the 2007 EFSA Conclusion (EFSA Scientific Report (2007) 106, 1-98) that were sufficient to fulfil the data requirements and guidance relevant at the time of the active substance inclusion, remain sufficient to support the product application.
The assessment should be revised when the active substance is renewed and the new methods should be provided by the applicant for re-evaluation.

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 in animal matrices is given in the following tables. No new studies have been submitted with this application.

Table 5.3-4: Validated methods for food and feed of plant origin (required for all matrix types, "difficult" matrix only when indicated by intended GAP)

Component of residue definition: prothioconazole-desthio, prothioconazole 3-hydroxy-desthio, prothioconazole 4-hydroxy-desthio				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Heinemann, O., 2001b, (DAR, 2005)
	ILV	0.004 mg/kg	HPLC-MS/MS	Heinemann, O., 2001c, (DAR, 2005)
Muscle	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Heinemann, O., 2001b, (DAR, 2005)
	ILV	0.004 mg/kg	HPLC-MS/MS	Heinemann, O., 2001c, (DAR, 2005)
Fat	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Heinemann, O., 2001b, (DAR, 2005)
	ILV	0.004 mg/kg	HPLC-MS/MS	Heinemann, O., 2001c, (DAR, 2005)
Kidney, liver	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Heinemann, O., 2001b, (DAR, 2005)
	ILV	0.004 mg/kg	HPLC-MS/MS	Heinemann, O., 2001c, (DAR, 2005)

Table 5.3-5: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	Heinemann, O., 2001b; Heinemann, O., 2001c (DAR, 2005)
Not required, because:	-

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in soil is given in the following table. No new studies have been submitted with this application.

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

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	6 µg/kg (prothioconazole and	HPLC-MS/MS	Schramel, O., 2000, (DAR, 2005)

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
	prothioconazole-desthio)		
Primary & confirmatory	10 µg/kg (prothioconazole-desthio)	LC-MS/MS	Steinhauer, S., 2001, (DAR, 2005)

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in surface and drinking water is given in the following tables. No new studies have been submitted with this application.

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

Component of residue definition: prothioconazole and prothioconazole-desthio				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water and surface water	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	Sommer, H., 2001b (DAR, 2005)

zRMS comment:
An independent laboratory validation (ILV) for drinking water is missing (required under Reg (EU) No 283/2013).
This data gap should 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.

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in air is given in the following tables. No new studies have been submitted with this application.

Table 5.3-8: Validated methods for air (if appropriate)

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary & confirmatory	0.015 mg/m^3	HPLC-MS/MS	Maasfeld, W., 2002a (DAR, 2005)
Primary	0.0006 mg/m^3	HPLC-MS/MS	Maasfeld, W., 2002b (DAR, 2005)

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

Methods for body fluids and tissues are not required, because prothioconazole is not considered to be toxic or very toxic (T / T+) nor is it classified according to CLP regulatory as follows: Acute toxicity (cat. 1 - 3), CMR (cat. 1) or STOT (cat. 1) (DAR, 2005).

zRMS:
- monitoring methods for body fluids and tissues (post-registration requirement – minor data gap; currently agreed EU endpoints for prothioconazole do not include a residue definition for 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.

5.3.2.8 Other studies/ information

Summary of validation of analytical methods used in dRR Section 9 (Ecotoxicology) and Section B7 (Metabolism and Residues) are provided in Appendix 2.

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	Łysik A.	2022	Protiokonazol 300 EC. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage. Report No BF – 25/22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri International Sp. z o.o.**
KCP 5.1.2/01	Peda T.	2022	Magnitude of residue of prothioconazole and metabolites prothioconazole-desthio, triazole alanine (TA), 1,2,4-triazole (1,2,4-T), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey after one application of Protiokonazol 300 EC on phacelia (Raw agricultural Commodity) – two harvest study trials in Poland. Study Code: 22SGS46; LBN-0044-2022 SGS Polska Sp. z o. o. GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/02	Mautino G.	2023	Earthworm Reproduction Test (*Eisenia fetida*) with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Validation of an analytical method and determination of content of PROTHIOCONAZOLE in soil samples (OECD 222) Study Code: 1139.1F.SAG22; Test Site Code: 22293-02R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2/03	Mautino G.	2023	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure Analytical Phase: Validation of an analytical method and determination of the content of PROTHIOCONAZOLE in the water stock solutions (OECD 239) Study Code: 1002.1F.SAG22; Test Site Code: 22293-03R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
	Morsiani S.	2023	Amendment No. 1 Analytical Phase Report 22293-03R (1002.1F.SAG22) Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure Analytical Phase: Validation of an analytical method and determination of the content of PROTHIOCONAZOLE in the water stock solutions (OECD 239)		
KCP 5.1.2/04	Mautino G.	2023	Predatory mites *Hypoaspis (Geolaelaps) aculeifer* reproduction test in soil with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of content of PROTHIOCONAZOLE in soil samples Study code: Study Code: 1142.1F.SAG22; Test Site Code: 22293-04R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/05	Mautino G.	2023	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Chronic Oral Toxicity Test Analytical Phase: Validation of an Analytical method and determination of content of prothioconazole in the feeding solutions of honey bees new born workers and in the water stock solution (OECD 245) Study Code: 1001.1F.SAG22; Test Site Code: 22293-01R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/06	Mautino G.	2023	Collembolan *Folsomia candida* reproduction test in soil with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of content of PROTHIOCONAZOLE in soil	N	Pestila Sp. z o.o.* ProAgri

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
			samples (OECD 232) Study Code: 1143.1F.SAG22; Test Site Code: 22293-05R Renolab S.r.l., Italy GLP Unpublished		Sp. z o.o.**
KCP 5.1.2/07	Artusio M.	2023	Daphnia sp. Acute Immobilization Test (*Daphnia magna*) with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of the content of PROTHIOCONAZOLE in water medium samples (OECD 202) Study Code: 1136.1F.SAG22; Test Site Code: 22293-06R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/08	Mautino G.	2023	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Bumblebee (*Bombus terrestris* L.) in the laboratory – Acute Oral and Contact Toxicity Test Analytical Phase: Determination of content of prothioconazole in the feeding solutions and in the water contact solution for bumblebees (OECD 246 and 247) Study Code: 1138.1F.SAG22; Test Site Code: 22293-07R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/09	Mautino G.	2023	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on terrestrial Non-target plants – Seedling Emergence and Seedling growth Analytical Phase: Determination of the content of PROTHIOCONAZOLE in the water spray solutions (OECD 208) Study Code: 1140.1F.SAG22; Test Site Code: 22293-08R Renolab S.r.l., Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
KCP 5.1.2/10	Mautino G.	2023	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Terrestrial Plant Vegetative Vigour – OECD 227 Analytical Phase: Determination of the content of PROTHIOCONAZOLE in the water spray solution	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**

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
			Study Code: 1141.1F.SAG22; Test Site Code: 22293-09R Renolab S.r.l., Italy GLP Unpublished		
KCP 5.1.2/11	Tediosi E.	2023	Validation of the analytical method for the determination of prothioconazole content in aqueous solutions coming from ecotoxicological tests with PROTIOKONAZOL 300 EC Study No. CH – 0912-2022 ChemService S.r.l. Controlli e Ricerche, Italy GLP Unpublished	N	Pestila Sp. z o.o.* ProAgri Sp. z o.o.**
			STUDY PLAN AMENDMENT No.1 Validation of the analytical method for the determination of prothioconazole content in aqueous solutions coming from ecotoxicological tests with PROTIOKONAZOL 300 EC Study No. CH – 0912-2022 ChemService S.r.l. Controlli e Ricerche, Italy GLP Unpublished		
	Longhi, D.	2021c	Validation of an analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw Report No. : 21-120 LabAnalysis s.r.l., Casanova Lonati – Italy GLP : Yes Unpublished	No	Indofil Letter of Access
	Longhi, D.	2021b	Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities	No	Indofil Letter of

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
			Report No. : 21-108 LabAnalysis s.r.l., Casanova Lonati – Italy GLP : Yes Unpublished Part of the study for which the access is granted: Validation of analytical method for determination of TDM (TRZ, TA, TLA, TAA) in Whole Plant (Rapeseed), wheat grain, white bread, straw		Access
	Longhi, D.	2021a	Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities Report No. : 21-31 LabAnalysis s.r.l., Casanova Lonati – Italy GLP : Yes Unpublished Part of the study for which the access is granted: Validation of analytical method for determination of Prothioconazoledesthio in Whole Plant (Rapeseed),wheat grain, white bread, straw	No	Indofil Letter of Access
	Nichetti, S.	2022a	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat) Report No. : CH-1081/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished Part of the study for which the access is granted: ILV of analytical method for determination of Prothioconazoledesthio in Straw (wheat)	No	Indofil Letter of Access
	Nichetti, S.	2022b	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of	No	Indofil

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**
			Difenoconazole and Prothio-desthio in Grain (Wheat) Report No.: CH-1082/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished Part of the study for which the access is granted: ILV of analytical method for determination of Prothioconazoledesthio in Grain (wheat)		Letter of Access
	Nichetti, S.	2022d	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed) Report No.: CH-1084/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished Part of the study for which the access is granted: ILV of analytical method for determination of Prothioconazoledesthio in Whole Plant (Rapeseed)	No	Indofil Letter of Access
	Nichetti, S.	2022g	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed) Report No.: CH-1085/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished	No	Indofil Letter of Access
	Nichetti, S.	2022i	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Straw (wheat) Report No.: CH-1086/2021	No	Indofil Letter of Access

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
			ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished		
	Nichetti, S.	2022h	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Grain (wheat) Report No.: CH-1087/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished	No	Indofil Letter of Access
	Nichetti, S.	2022e	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Prothio-desthio metabolites in Cereal straw Report No.: CH-1091/2021 ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy GLP : Yes Unpublished	No	Indofil Letter of Access

*Pestila Spółka z ograniczoną odpowiedzialnością (short name: Pestila Sp. z o.o.)
**ProAgri Spółka z ograniczoną odpowiedzialnością / ProAgri International Spółka z ograniczoną odpowiedzialnością (short names: ProAgri Sp. z o.o. / ProAgri International Sp. z o.o.)

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

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

The following tables are to be completed by MS

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for prothioconazole

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

Please refer to the points 5.2.1.1 and 5.2.1.2.

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 A.2.A.9 Other Studies/ Information

A 2.1.2.7.1 HPLC-MS/MS (in honey)

A 2.1.2.7.1.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/01
Report	Magnitude of residue of prothioconazole and metabolites prothioconazole-desthio, triazole alanine (TA), 1,2,4-triazole (1,2,4-T), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey after one application of Protiokonazol 300 EC on phacelia (Raw agricultural Commodity) – two harvest study trials in Poland, Study Code: 22SGS46; LBN-0044-2022, Peda T., 2022
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Below described methods are also suitable and were use for purpose of the study: "Magnitude of the residue of prothioconazole, prothioconazole-desthio and triazole-derivative metabolites (TDMs) in honey after one application of PROTIOKONAZOL 300 EC on Phacelia crop under semi field conditions in two trials in Southern Europe – 2022"; Study code: 1147.2F.SAG22; LBN-0045-2022; Rovetto I., 2022)

VALIDATION OF THE ANALYTICAL METHOD FOR THE QUANTIFICATION OF PROTHIOCONAZOLE AND PROTHIOCONAZOLE-DESTHIO

Principle of the method

The analytical method to quantify prothioconazole and its metabolite prothioconazole-desthio in honey was based in a dissolution of the sample in a mixture of acetonitrile/water 80:20 containing 5 g/L of L-cysteine hydrochloride. Then, the separation of the acetonitrile layer was achieved by the addition of sodium chloride. After centrifugation and filtration, the acetonitrile extract was analysed using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry), acquiring 2 simultaneous MRM transitions (primary and confirmatory) for each analyte. The validation of the analytical method was carried out under GLP compliance according to SANTE/2020/12830 rev.1 guideline. The evaluated validation parameters were: matrix effect, calibration, accuracy and precision limit of quantification and detection, selectivity, confirmatory, stability of final extracts and standard solutions.

Materials and methods

Chromatograph:	HPLC Agilent 1290 Infinity II + triple quadrupole mass spectrometer Agilent 6470A
Analytical column:	Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Injection volume:	2.5 µl
Column temperature:	40°C
Mobile phase A:	LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate
Mobile phase B:	LC-MS grade methanol with 0.2% formic acid 5 mM ammonium formate
Flow:	0.6 mL/min
Gradient elution:	

Time (min)	%B
0	50
0.50	50

3.00	100
5.00	100

Stop time: 5 min
Post time: 1.5 min
Source type: ESI
Gas temperature: 350°C
Gas flow: 8 L/min
Nebulizer: 40 psi
Sheath gas heater: 400°C
Sheath gas flow: 12 L/min
Capillary: 3500 V
Vcharging: 0 V
Acquiring mode: ESI positive, MRM (multi-reaction monitoring)

Validation

Selectivity
This parameter was evaluated in order to demonstrate that the applied method detects the right analytes and that the analytical signals are quantitatively correct and not affected by other analytes or by the matrix.
Using a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: an untreated sample, a fortified sample and a reference solution at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals were detected in the untreated sample in amounts higher than 30% of the LOQ level which is in compliance with the guideline requirements. The method was found selective for the determination of the analytes Prothioconazole and Prothioconazole-desthio in the tested honey for both the monitored transitions.

Calibration
The analytical calibrations were performed by comparing the injected amounts of the analytical standard solutions and the corresponding analytes peak areas. Linearity was checked by 5-points calibration curves (single injections) using matrix-matched standard solutions.
The equations of the calibration curves were calculated with the minimum square linear regression method (1/x weighed).

Matrix	**Analyte**	**Range (µg/L)**	**Range**	**R^2 primary transition**	**R^2 confirmatory transition**
Honey	Prothioconazole	0.500 – 50.0 (5 concentration levels in single injections, covering two orders of magnitude)	0.0020 mg/kg – 0.200 mg/kg (20% LOQ – 100% above 10xLOQ)	0.99886785	0.99943490
	Prothioconazole-desthio			0.99966065	0.99950985

The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration. Furthermore, the obtained values of R^2 are > 0.99. Therefore, the calibration is considered linear in the explored concentration range.

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

- LOQ level (5 replicates): 0.01 mg/kg for each analyte
- 10xLOQ level (5 replicates): 0.1 mg/kg for each analyte

The mean recovery and relative standard deviation (RSD) per level found for both primary and confirmatory transitions were in compliance with the following requirements:

- LOQ level:
  - Range of recovery: 60-120%
  - RSD: < 30%
- 10xLOQ level:
  - Range of recovery: 70-120%
  - RSD: < 20%.

Matrix Effect

Assessment of matrix effect was performed by comparing the analytes responses of one standard at L5 (50 µg/L of each analyte) prepared in solvent to one prepared in blank matrix at the same concentration.

The matrix effect is not exceed ± 20 %. The matrix effects are presented in table below.

Matrix	Analyte	Analyte area in L5 standard in matrix	Analyte area in L5 standard in solvent	Area matrix-matched/ Solvent %	Matrix effect [%]
Honey	Prothioconazole	2829	2891	97.9	-2.1
	Prothioconazole-desthio	55169	62820	87.8	-12.2

Limit of detection (LOD)

The Limit of Detection (LOD) is the smallest concentration at which an analyte produces an instrumental signal at least 3 times higher than the background noise of the chromatogram. It should not be higher than 30% of the LOQ value. The lowest concentrated matrix-matched standard injected can be considered the limit of detection. This level corresponds to 20% of the target LOQ for each analyte which is in compliance with the limit of 30% of the LOQ required by the guideline.

Matrix	Analyte	LOD Concentration	% of LOQ	Transition	S/N at LOD level
Honey	Prothioconazole	0.500 µg/L (0.002 mg/kg)	20	344.0/125.0 (primary)	5.8
				344.0/102.3 (confirmatory)	3.2
	Prothioconazole-desthio	0.500 µg/L (0.002 mg/kg)	20	312.2/69.8 (primary)	41.5
				312.2/125.0 (confirmatory)	300.2

Limit of quantification (LOQ)

The Limit of quantification (LOQ) is defined as the lowest concentration at which an acceptable recovery is obtained. The target LOQ for this study was set to 0.01 mg/kg for each analyte, that was the lowest concentration levels tested during the recovery tests. At these concentrations, the established requirements for mean recovery and precision were 60-120% and %RSD ≤ 30%, respectively. From the elaboration of the obtained data, recovery and precision (as repeatability) resulted in compliance with the requirements for both the analytes. This means that the LOQ was successfully verified.

Untreated sample analysis

Two analytical untreated sub-samples were analysed, according to the analytical method, in order to assess the absence of Prothioconazole and Prothioconazole-desthio. The results obtained are reported in the following table:

Matrix	Sample code	Measured Prothioconazole concentration (mg/kg)		Measured Prothioconazole-desthio concentration (mg/kg)	
		Primary transition (344.0/125.0)	Confirmatory transition (344.0/102.3)	Primary transition (312.2/69.8)	Confirmatory transition (312.2/125.0)

Honey	22-44-P-01	< LOD	< LOD	< LOD	< LOD
	22-44-P-02	< LOD	< LOD	< LOD	< LOD

Confirmatory
A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring additional SRM transitions.
Product ions scans of a 1 mg/L solution (Solution B) was carried out to justify the selection of the MS/MS transitions used:

- Prothioconazole: fragmentation of the molecular ion 344.0 m/z to give the fragments 125.0 and 102.3
- Prothioconazole-desthio: fragmentation of the molecular ion 312.2 m/z to give the fragments 69.8 and 125.0

Stability of final extracts and standard solutions
Stability of standard solutions
In order to check the stability of Prothioconazole and Prothioconazole-desthio in the stock solutions prepared in acetonitrile, the instrumental responses obtained from 5 replicated analysis of diluted solutions were evaluated.
The stock solutions identified as "old" were used to prepare a solution at 100 µg/L. A solution at about the same concentration (identified as "new") was prepared 29 days after the preparation of the first stock solutions, in the same way. Each diluted solution was then analysed in 5 replicates, recording the areas and comparing the mean values of the response factors.
Since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly prepared stock solutions was < 10%, it is possible to assess that the analytes Prothioconazole and Prothioconazole-desthio are stable in the stock solutions prepared in acetonitrile for at least 29 days if stored in the dark at 5 ± 3°C.

Stability of final extracts
In order to check the stability of Prothioconazole and Prothioconazole-desthio in the final extract, aliquots of untreated samples extracts were spiked with known amounts of analyte at the concentration of L5 calibration level. The stability in the extracts was tested for a period of 3 days at 5 ± 3°C in dark conditions: after the storage period, the stored extracts were analysed concurrently with the same matrix-matched standard solution freshly prepared, used as reference for time 0. The measured instrumental responses were compared and the stability was expressed as the percentage ratio between the responses of the spiked stored extract and the freshly spiked one.

Matrix	**Analyte**	**Storage conditions**	**Area of analyte in untreated matrix extract (L5 level)**		**Stability (area stored/area t_0) %**
			time 0	**after storage**	
Honey	Prothioconazole	3 days at 5 ± 3°C	16980	16080	94.7
	Prothioconazole-desthio		71525	70623	98.7

The stability of the analytes in the extract can be considered proven for at least 3 days at 5 ± 3°C in the dark since the recoveries of the stored spiked samples are within the range of 70-120% measured against the freshly prepared ones, as required by the SANTE/2020/12830 rev.1 guideline.

VALIDATION OF THE ANALYTICAL METHOD FOR THE QUANTIFICATION OF TDM

Principle of the method

The applied analytical method (AM2-LBN-0044-2022) allow the determination of the following TDM

(triazole-derivative metabolites): 1,2,4-triazole (TRZ), Triazole-alanine (TA), Triazole-lactic acid (TLA), Triazole-acetic acid (TAA).
After addition of a proper amount of ILIS (isotope-labelled internal standards), the honey sample was dissolved in deionised water. After that, methanol with 1 % formic acid was added. The mixture was then filtered and analysed using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS) acquiring 2 simultaneous MRM transitions (primary and confirmatory) for each analyte. The validation of the analytical method was carried out under GLP compliance according to SANTE/2020/12830 rev.1 guideline. The evaluated validation parameters were: matrix effect, calibration, accuracy and precision limit of quantification and detection, selectivity, confirmatory, stability of final extracts and standard solutions.

Materials and methods

Chromatograph:	HPLC Shimadzu LC-40 XR + Sciex API 6500 + equipped with Selex-ION+ (Differential Ion Mobility Device)
Analytical column:	Thermo Hypercarb 5 µm, 2.1 x 100 mm
Injection volume:	2 µl
Column temperature:	40°C
Mobile phase A:	LC-MS grade water with 1% acetic acid
Mobile phase B:	LC-MS grade methanol with 1% acetic acid
Flow:	0.6 mL/min

Gradient elution:

Time (min)	%A	%B
0	100	0
1	100	0
6	10	90
7	10	90
7.1	100	0

Stop time:	10 min
Source type:	ESI
Gas temperature:	500°C
Curtain gas flow:	30 L/min
Gas 1:	55 mL/min
Gas 2:	65 mL/min
Capillary:	positive mode 3500 V
Acquiring mode:	ESI positive, MRM (multi-reaction monitoring)

Validation

Selectivity
This parameter was evaluated in order to demonstrate that the applied method detects the right analytes and that the analytical signals are quantitatively correct and not affected by other analytes or by the matrix.
Using a MS/MS mass spectrometer detector equipped with a DMS device, the selectivity was evaluated comparing the following chromatograms: an untreated sample, a fortified sample and a reference solution at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals were detected in the untreated sample in amounts higher than 30% of the LOQ level which is in compliance with the guideline requirements. The method was found selective for the determination of the analytes Prothioconazole and Prothioconazole-desthio in the tested honey for both the monitored transitions.

Calibration
The analytical calibrations were performed by comparing the injected amounts of the analytical standard solutions and the corresponding analytes peak areas. Linearity was checked by 5-points calibration curves (single injections) using standard solutions in solvent (water / 1% formic acid in methanol 50:50).
The equations of the calibration curves were calculated with the minimum square linear regression meth-

od (1/x weighed).

Matrix	Analyte	Range (µg/L)	Range	R^2 primary transition	R^2 confirmatory transition
Honey	1,2,4-Triazole	Each analyte: 0.500 – 50.0 (5 concentration levels in single injections, covering two orders of magnitude)	Each analyte: 0.0020 mg/kg – 0.200 mg/kg (20% LOQ – 100% above 10xLOQ)	0.9967426569	0.9979210816
	Triazole Alanine			0.99740169	0.9960439204
	Triazole Acetic Acid			0.9998200081	0.9999000025
	Triazole Lactic acid			0.9990202401	0.9986204761

The regression residuals plots show that residuals are randomly distributed, hence demonstrating the linear calibration. Furthermore, the obtained values of R^2 are > 0.99. Therefore, the calibration is considered linear in the explored concentration range.

Accuracy and precision

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

- LOQ level (5 replicates): 0.01 mg/kg for each analyte
- 10xLOQ level (5 replicates): 0.1 mg/kg for each analyte

The mean recovery and relative standard deviation (RSD) per level found for both primary and confirmatory transitions were in compliance with the following requirements:

- LOQ level:
  - Range of recovery: 60-120%
  - RSD: < 30%
- 10xLOQ level:
  - Range of recovery: 70-120%
  - RSD: < 20%.

Matrix Effect

Assessment of matrix effect was performed by comparing the analytes responses of one standard at L5 (50 µg/L of each analyte) prepared in solvent to one prepared in blank matrix at the same concentration.

The matrix effect is not exceed ± 20 %. The matrix effects are presented in table below.

Matrix	Analyte	Compound	Matrix-matched standard L5		Standard solvent L5		Ratio matrix-matched/ Solvent %	Δ%
			area	area ratio (analyte/ISTD)	area	area ratio (analyte/ISTD)		
Honey	1,2,4-Triazole	Analyte	30803	3.0252	58143	3.2088	94.3	-5.7
		ISTD	10182	-	18120	-		
	Triazole Alanine	Analyte	555726	47.6405	537364	52.2778	91.1	-8.9
		ISTD	11665	-	10279	-		
	Triazole Acetic Acid	Analyte	656728	4.5273	915548	4.57925	98.9	-1.1
		ISTD	145058	-	199934	-		
	Triazole Lactic Acid	Analyte	358525	3.5648	467590	3.7248	95.7	-4.3
		ISTD	100575	-	125533	-		

Limit of detection (LOD)

The Limit of Detection (LOD) is the smallest concentration at which an analyte produces an instrumental signal at least 3 times higher than the background noise of the chromatogram. It should not be higher than 30% of the LOQ value. The lowest concentrated matrix-matched standard injected can be considered the limit of detection. This level corresponds to 20% of the target LOQ for each analyte which is in compli-

ance with the limit of 30% of the LOQ required by the guideline.

Matrix	Analyte	LOD Concentration	% of LOQ	Transition	S/N at LOD level
Honey	1,2,4-triazole	0.500 µg/L (0.002 mg/kg)	20	70.1/43.1 (primary)	10
				70.1/70.0 (confirmatory)	10.7
	Triazole-alanine	0.500 µg/L (0.002 mg/kg)	20	157/70 (primary)	32.9
				157/88 (confirmatory)	34.3
	Triazole-acetic acid	0.500 µg/L (0.002 mg/kg)	20	128/70 (primary)	125.3
				128/73 (confirmatory)	12.1
	Triazole-lactic acid	0.500 µg/L (0.002 mg/kg)	20	158/70 (primary)	23
				158/43 (confirmatory)	10.9

Limit of quantification (LOQ)
The Limit of quantification (LOQ) is defined as the lowest concentration at which an acceptable recovery is obtained. The target LOQ for this study was set to 0.01 mg/kg for each analyte, that was the lowest concentration levels tested during the recovery tests. At these concentrations, the established requirements for mean recovery and precision were 70-120% and %RSD ≤ 20%, respectively. From the elaboration of the obtained data, recovery and precision (as repeatability) resulted in compliance with the requirements for both the analytes. This means that the LOQ was successfully verified.

Untreated sample analysis
Two analytical untreated sub-samples were analysed, according to the analytical method, in order to assess the absence of Prothioconazole and Prothioconazole-desthio. The results obtained are reported in the following tables.

Matrix	Sample code	Measured 1,2,4-triazole concentration (mg/kg)		Measured Triazole-alanine concentration (mg/kg)	
		Primary transition (70.1/43.1)	Confirmatory transition (70.1/70)	Primary transition (157/70)	Confirmatory transition (157/88)
Honey	22-44-P-01	< LOD	< LOD	< LOD	< LOD
	22-44-P-02	< LOD	< LOD	< LOD	< LOD

Matrix	Sample code	Measured Triazole-acetic acid concentration (mg/kg)		Measured Triazole-lactic acid concentration (mg/kg)	
		Primary transition (128/70)	Confirmatory transition (128/73)	Primary transition (158/70)	Confirmatory transition (158/43)
Honey	22-44-P-01	< LOD	< LOD	< LOD	< LOD
	22-44-P-02	< LOD	< LOD	< LOD	< LOD

Confirmatory
A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring additional SRM transitions.
Product ions scans of a 1 mg/L solution (Solution B) was carried out to justify the selection of the MS/MS transitions used:

- 1,2,4-triazole: fragmentation of the molecular ion 70.1 m/z to give the fragments 43.1 and 70.0
- Triazole-alanine: fragmentation of the molecular ion 157 m/z to give the fragments 70 and 88
- Triazole-acetic acid: fragmentation of the molecular ion 128 m/z to give the fragments 70 and 73
- Triazole-lactic acid: fragmentation of the molecular ion 158 m/z to give the fragments 70 and 43

Stability in sample extracts
According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (ILIS) for quantification, since the ILIS compensates for losses during extract storage. Since ILIS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.

Stability of standard solutions
In order to check the stability of 1,2,4-triazole (TRZ), triazole-alanine (TA), triazole-lactic acid (TLA) and triazole-acetic acid (TAA) in the stock solutions prepared in water, the instrumental responses obtained from 5 replicated analyses of diluted solutions were evaluated.
The stock solutions identified as "old" were used to prepare a solution at 100 µg/L. A solution at about the same concentration (identified as "new") was prepared 29 days after the preparation of the first stock solutions, in the same way. Each diluted solution was then analysed in 5 replicates, recording the areas and comparing the mean values of the response factors.
Since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 29 days, if stored in the dark at 5 ± 3°C.

Conclusion

The methods were fully validated. Results of the validation of analytical methods was confirmed that these methods are suitable for analysis the content of the test items in matrix (honey) in residue studies.

A 2.1.2.7.2 HPLC - LC-MS/MS (in soil)

A 2.1.2.7.2.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/02
Report	Earthworm Reproduction Test (*Eisenia fetida*) with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L), Analytical Phase: Validation of an analytical method and determination of content of PROTHIOCONAZOLE in soil samples (OECD 222), Study Code: 1139.1F.SAG22, Test Site Code: 22293-02R, Mautino G., 2023
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in soil samples collected during the biological phase of the study.
The analytical method for prothioconazole in soil was fully validated during the study according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was extracted from soil samples with methanol and acidified water mixtures; the final

analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Equipment

- Standard laboratory glassware and equipment
- Analytical electronical balance with 0.1 mg accuracy, AT 261, METTLER TOLEDO
- Technical electronic balance with 0.01 g accuracy PS 2100.R2 , RADWAG
- Automatic pipette 2-20 µL, Pipetman, GILSON
- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Ultrasonic bath 2210, BRANSON
- Horizontal shaker with heating bath, H20S, LAUDA
- Centrifuge Megafuge 16, HERAEUS
- Water purification system Direct-Q 3UV, MILLIPORE
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCI-EX
- Thermostatic oven, model 11120-VF, MPM Instruments

Reagents and materials

- MilliQ Water from Direct-Q 3UV MILLIPORE
- Acetonitrile for UHPLC-MS, CARLO ERBA
- Methanol for UHPLC-MS, CARLO ERBA
- Formic acid ≥ 95%, SIGMA ALDRICH
- Acetic acid glacial ≥ 99.9%, CARLO ERBA
- Syringe PTFE filters 0.22 µm, 13 mm diameter, VWR
- Single use syringes
- 1 % acetic acid in water
- Methanol / 1 % acetic acid in water 80/20 solvent mixture
- Methanol / 1 % acetic acid in water 50/50 solvent mixture
- Eluent A: Water (milliQ), 0.1% formic acid
- Eluent B: Acetonitrile (UHPLC-MS), 0.1% formic acid

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile (UHPLC-MS), 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: ~5.5 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi

DP: 28
EP: 2.4

- Transitions: PROTHIO-1_T (344/326) CE 5 , CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53 , CXP 2.4, dwell time 200 msec

Analytical procedure for soil samples analysis

The sample was let to warm up to room temperature.
The homogenised sample (6.00 ± 0.05 g) was weighted into a 50 mL centrifuge tube, recovery samples were fortified at this point.
Then 10 mL of methanol were added, the sample was sonicated for 5 minutes, manually shaken for a few seconds to homogenise, and shaken for 30 minutes with a horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer collected into a centrifuge tube.
The soil is further extracted with 8 mL of a mix methanol/1% acetic acid in water 80/20, then manually shaken for a few seconds to homogenise and shaken for 5 minutes by horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
The soil is extracted a third time with 7 mL of a mix methanol/1% acetic acid in water 50/50, then manually shaken for a few seconds to homogenise and shaken for 5 minutes with a horizontal shaker. Finally, the sample was centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
All the extracts, combined in the centrifuge tube, were brought to a final volume of 25 mL with 1% acetic acid in water, then an aliquot of the extract was filtered on 0.22 µm PTFE membrane into a vial for the analysis.
Samples with analyte concentration exceeding 80 ng/mL were opportunely diluted with blank untreated extract in order to fall within ± 20 % of the calibration range.
Recovery samples at second level were diluted 1:10000 with blank untreated extract in order to obtain final extract concentrations falling within ± 20 % of the calibration range.
The samples were analysed by high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS).
Quantification was conducted using an external standard calibration curve obtained by linear regression of matrix matched calibration standards injected throughout the run in the range 2.5 – 100 ng/mL.

Validation

Blank and selectivity
Four independent analyses of the blank sample were performed: no significant interference exceeding 30% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated soil sample used in fortification trials (< 30% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in soil matrix, without significant interferences above 30% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 2.5 - 100 ng/mL corresponding to 0.014 to 0.56 mg/kg of prothioconazole in dry soil sample. The correlation coefficient of the weighed linear (1/x) multipoint external matrix matched standard calibration curves was found ≥ 0.995 for both ion transitions

in all the analytical sequences performed.
The linearity range comprised the concentration range from 30% of the LOQ to at least 20% above the highest measured concentration.

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

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

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

Recoveries
The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery was calculated.
The recoveries were performed by fortifying the untreated blank at two levels.
The LOQ level was set at prothioconazole concentration of 0.05 mg/kg $_{\text{dry weight}}$ (lower than the minimum found prothioconazole content in samples, while the second level was at 2024 mg/kg dry weight (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.
The background content in the control sample used for fortification experiments was not detectable. In these recovery samples the prothioconazole content was determined as reported below.

Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	74.4 88.9 70.4 73.8 85.7	78.6	10.3	80.2 ± 10.2
2024 (II level)	68.6 88.3 86.5 88.5 76.6	81.7	10.8	

DW = Dry Weight

For each fortification level the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy
The accuracy of the analysis method for prothioconazole in soil, defined as mean recovery ± relative standard deviation, is 80.2 ± 10.2.

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

Fortification level (mg/kg DW)	RSD % (n = 5)	Overall RSD % (n = 10)
0.05 (LOQ)	10.3	10.2
2024 (II level)	10.8	

DW = Dry Weight

Matrix Effect

To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of prothioconazole at 10 ng/mL (nearest to the nominal concentration for the LOQ level) was compared to prothioconazole in solvent at the same concentration. The results are summarized in the following table.

Transition	Matrix response over solvent response %	Matrix effects %
344/326 (Target)	111	+ 11
344/125 (Qualifier)	107	+ 7

Matrix effects for prothioconazole in soil matrix were found not significant (< 20%) for both acquired transitions, nevertheless matrix matched calibration standards were used in the quantification of samples for better accuracy.

Limit of detection (LOD) and limit of quantification (LOQ)

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

For the analysis of prothioconazole in soil the LOD is 0.014 mg/kg (referred to dry soil). This value, calculated from the prothioconazole concentration corresponding to the lowest calibration point, is below 30% of LOQ.

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

The LOQ for prothioconazole in soil was assessed in this study at 0.05 mg/kg (referred to dry soil).

Confirmation

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

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

Fortification level (mg/kg DW)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.05 (LOQ)	72.5 87.7 78.1 71.8 86.1	79.2	9.4	80.6 ± 10.7
2024 (II level)	64.9 87.4 90.6 87.2 80.0	82.0	12.6	

DW = Dry Weight

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions
During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.
The final extracts were analysed within 24 hours form extraction. Moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.1.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in soil samples in the range 0.05 – 2024 mg/kg $_{DW}$.
As the analysis of the samples was performed concurrently with the recovery tests, the reliability of the found values was demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (mg/kg $_{DW}$)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	74.4 88.9 70.4 73.8 85.7	78.6	10.3	80.2 ± 10.2
2024 (II level)	68.6 88.3 86.5 88.5 76.6	81.7	10.8	
Limits of the method Limit of quantification: 0.05 mg/kg Limit of detection: 0.014 mg/kg Linearity range: from 2.5 to 100 ng/mL (corresponding to 0.014 - 0.56 mg/kg in undiluted samples) r ≥ 0.995 Method validation range: 0.05 – 2024 mg/kg $_{DW}$				

DW = Dry Weight

A 2.1.2.7.3 HPLC - LC-MS/MS (in water)

A 2.1.2.7.3.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference: KCP 5.1.2/03

Report	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure Analytical Phase: Validation of an analytical method and determination of the content of PROTHIOCONAZOLE in the water stock solutions (OECD 239), Mautino G., 2023, Study Code: 1002.1F.SAG22; Test Site Code: 22293-03R Amendment No. 1 Analytical Phase Report 22293-03R (1002.1F.SAG22) Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure Analytical Phase: Validation of an analytical method and determination of the content of PROTHIOCONAZOLE in the water stock solutions (OECD 239)
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in the lowest concentration and in the highest concentration of the water stock solutions prepared in the biological phase of the study.
The analytical method for prothioconazole in water was fully validated in this study according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was determined in water stock solutions after sample dilution with acetonitrile and the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Equipment

- Standard laboratory glassware and equipment
- Analytical electronical balance with 0.1 mg accuracy, AT 261, METTLER TOLEDO
- Automatic pipette 2-20 µL, Pipetman, GILSON
- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Water purification system Direct-Q 3UV, MILLIPORE
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCIEX

Reagents and materials

- Acetonitrile for HPLC gradient grade, HONEYWELL ≥ 99.9%
- MilliQ Water from Direct-Q 3UV MILLIPORE
- Formic acid 98-100%, SIGMA ALDRICH
- Syringe PTFE filters 0.2 µm, 13 mm diameter, AGILENT
- Single use syringes

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent

- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile, 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: 4.8-5.5 minutes*
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5 , CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53 , CXP 2.4, dwell time 200 msec

** two analytical columns of the same type were used during the study: slight differences in retention times are due to the different batches of the two columns stationary phase*

Analytical procedure for sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately 1 mL is taken and brought to 5 mL with acetonitrile.
Recovery samples were prepared in water using the test item.
Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
Recovery samples at second level for matrix water were diluted 1:2000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The extract was finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.

Validation

Blank and selectivity
Two independent analyses of the blank sample were performed: no significant interference exceeding 10% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated water sample used in fortification trials (< 10% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in water solution matrix, without significant interferences above 10% of LOQ (taking into account the operative dilution of LOQ level).

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.

The mass spectrum of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision

The linearity range for prothioconazole was found between 10 - 1000 ng/mL corresponding to the range from 0.00005 to 0.005 g/L of prothioconazole in water samples. The correlation coefficient of the weighed linear (1/x) multipoint external standard solvent calibration curve was found ≥ 0.997 in the analytical sequence performed.
The linearity range comprised the concentration range from 10% of the LOQ (taking into account the operative dilution of LOQ level) to at least 20% above the highest measured concentration.

The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.
The recoveries were performed by fortifying the untreated blank at two levels.
The LOQ level was set at prothioconazole concentration of 0.00025 g/L (0.00085 g/L as test item), lower than the minimum found prothioconazole content in samples, while the second level was at 5.05 g/L (17 g/L as test item), higher than the maximum expected concentration in the samples, in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; six replicated analyses were carried out for each fortification level.
The background content in the control sample used for dilution of test item in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	114.0 120.1 111.6 117.3 118.1 84.1	110.9	12.1	108.2 ± 13.6
5.05 (b) (II level)	112.2 114.4 112.4 109.4 112.8 71.8	105.5	15.7	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

For each fortification level the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy
The accuracy of the analysis method for prothioconazole in water, defined as mean recovery ± relative standard deviation, is 108.2 ± 13.6.

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

Fortification level (g/L)	**RSD % (n = 6)**	**Overall RSD % (n = 12)**
0.00025 (a) (LOQ)	12.1	13.6
5.05 (b) (II level)	15.7	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

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

Transition	**Matrix response over solvent response %**	**Matrix effects %**
344/326 (Target)	117	+ 17
344/125 (Qualifier)	86	- 14

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

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in water the LOD is 0.00005 g/L. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point (undiluted sample).

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in water was assessed in this study at 0.00025 g/L.

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	122.2 118.5 112.8 114.6	109.8	11.4	106.1 ± 13.1

	116.1			
5.05 (b) (II level)	109.4 111.0 108.4 105.7 108.2	102.3	15.0	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions
The final extracts were analysed within 24 hours form extraction; moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The stability of prothioconazole reference item stock solution was verified to be stable for 36 days after preparation at ≤ - 18°C in the dark: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in water samples in the range 0.00025 – 5.05 g/L (corresponding to 0.00085 – 17 g/L as test item).
As the analysis of the samples was performed during the recovery tests, the reliability of the found values is demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	114.0 120.1 111.6 117.3 118.1 84.1	110.9	12.1	108.2 ± 13.6
5.05 (b) (II level)	112.2 114.4 112.4 109.4 112.8 71.8	105.5	15.7	
Limits of the method Limit of quantification: 0.00025 g/L Limit of detection: 0.00005 g/L Linearity range: from 10 to 1000 ng/mL (corresponding to 0.00005 - 0.005 g/L in undiluted samples) $r \geq 0.997$ Method validation range: 0.00025 – 5.05 g/L				

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

A 2.1.2.7.4 HPLC - LC-MS/MS (in soil)

A 2.1.2.7.4.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/04
Report	Predatory mites *Hypoaspis (Geolaelaps) aculeifer* reproduction test in soil with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of content of PROTHIOCONAZOLE in soil samples, Mautino G., 2023, Study Code: 1142.1F.SAG22; Test Site Code: 22293-04R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in soil samples collected during the biological phase of the study.
The analytical method for prothioconazole in soil was fully validated in Renolab analytical phase 22293-02R (Sagea Study code 1139.1F.SAG22) according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
In the current study the analytical method was reconfirmed in soil by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was extracted from soil samples with methanol and acidified water mixtures; the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Equipment

- Standard laboratory glassware and equipment
- Analytical electronical balance with 0.1 mg accuracy, AT 261, METTLER TOLEDO
- Technical electronic balance with 0.01 g accuracy PS 2100.R2, RADWAG
- Automatic pipette 2-20 µL, Pipetman, GILSON
- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Ultrasonic bath 2210, BRANSON
- Horizontal shaker with heating bath, H20S, LAUDA
- Centrifuge Megafuge 16, HERAEUS
- Water purification system Direct-Q 3UV, MILLIPORE
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCIEX
- Thermostatic oven, model 11120-VF, MPM Instruments

Reagents and materials

- MilliQ Water from Direct-Q 3UV MILLIPORE
- Acetonitrile for UHPLC-MS, CARLO ERBA
- Methanol for UHPLC-MS, CARLO ERBA
- Formic acid ≥ 95%, SIGMA ALDRICH
- Acetic acid glacial ≥ 99.9%, CARLO ERBA

- Syringe PTFE filters 0.22 µm, 13 mm diameter, VWR
- Single use syringes
- 1 % acetic acid in water
- Methanol / 1 % acetic acid in water 80/20 solvent mixture
- Methanol / 1 % acetic acid in water 50/50 solvent mixture
- Eluent A: Water (milliQ), 0.1% formic acid
- Eluent B: Acetonitrile (UHPLC-MS), 0.1% formic acid

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile (UHPLC-MS), 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: ~5.5 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for soil samples analysis

The sample was let to warm up to room temperature.
The homogenised sample (6.00 ± 0.05 g) was weighted into a 50 mL centrifuge tube, recovery samples were fortified at this point.
Then 10 mL of methanol were added, the sample was sonicated for 5 minutes, manually shaken for a few seconds to homogenise, and shaken for 30 minutes with a horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer collected into a centrifuge tube.
The soil is further extracted with 8 mL of a mix methanol/1% acetic acid in water 80/20, then manually shaken for a few seconds to homogenise and shaken for 5 minutes by horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
The soil is extracted a third time with 7 mL of a mix methanol/1% acetic acid in water 50/50, then manually shaken for a few seconds to homogenise and shaken for 5 minutes with a horizontal shaker. Finally, the sample was centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
All the extracts, combined in the centrifuge tube, were brought to a final volume of 25 mL with 1% acetic acid in water, then an aliquot of the extract was filtered on 0.22 µm PTFE membrane into a vial for the analysis.
Samples with analyte concentration exceeding 80 ng/mL were opportunely diluted with blank untreated extract in order to fall within ± 20% of the calibration range.

Recovery samples at second level were diluted 1:10000 with blank untreated extract in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The samples were analysed by high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS).
Quantification was conducted using an external standard calibration curve obtained by linear regression of matrix matched calibration standards injected throughout the run in the range 2.5 – 100 ng/mL.

Validation

Blank and selectivity
Four independent analyses of the blank sample were performed: no significant interference exceeding 30% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated soil sample used in fortification trials (< 30% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in soil matrix, without significant interferences above 30% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 2.5 - 100 ng/mL corresponding to 0.014 to 0.56 mg/kg of prothioconazole in dry soil sample. The correlation coefficient of the weighed linear (1/x) multipoint external matrix matched standard calibration curves was found ≥ 0.995 for both ion transitions in all the analytical sequences performed.
The linearity range comprised the concentration range from 30% of the LOQ to at least 20% above the highest measured concentration.

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

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

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

Recoveries
The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery was calculated.
The recoveries were performed by fortifying the untreated blank at two levels.
The LOQ level was set at prothioconazole concentration of 0.05 mg/kg $_{\text{dry weight}}$ (lower than the minimum found prothioconazole content in samples, while the second level was at 2027 mg/kg $_{\text{dry weight}}$ (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.
The background content in the control sample used for fortification experiments was not detectable. In

these recovery samples the prothioconazole content was determined as reported below.

Fortification level (mg/kg DW)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.05 (LOQ)	78.7 78.6	78.7	0.1	81.6 ± 4.3
2027 (II level)	85.4 83.8	84.6	1.3	

DW = Dry Weight

For each fortification level the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy
The accuracy of the analysis method for prothioconazole in soil, defined as mean recovery ± relative standard deviation, is 81.6 ± 4.3.

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

Fortification level (mg/kg DW)	RSD % (n = 2)	Overall RSD % (n = 4)
0.05 (LOQ)	0.1	4.3
2027 (II level)	1.3	

DW = Dry Weight

Matrix Effect
To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of prothioconazole at 10 ng/mL (nearest to the nominal concentration for the LOQ level) was compared to prothioconazole in solvent at the same concentration. The results are summarized in the following table.

Transition	Matrix response over solvent response %	Matrix effects %
344/326 (Target)	98	- 2
344/125 (Qualifier)	88	- 12

Matrix effects for prothioconazole in soil matrix were found not significant (< 20 %) for both acquired transitions, nevertheless matrix matched calibration standards were used in the quantification of samples for better accuracy.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in soil the LOD is 0.014 mg/kg (referred to dry soil). This value, calculated from the prothioconazole concentration corresponding to the lowest calibration point, is below 30% of LOQ.

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in soil was assessed in this study at 0.05 mg/kg (referred to dry soil).

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	71.6 75.6	73.6	3.8	78.7 ± 7.8
2027 (II level)	83.3 84.4	83.9	0.9	

DW = Dry Weight

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions
During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.
The final extracts were analysed within 24 hours form extraction. Moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.1.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in soil samples in the range 0.05 – 2027 mg/kg dry weight.
As the analysis of the samples was performed concurrently with the recovery tests, the reliability of the found values was demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	78.7 78.6	78.7	0.1	81.6 ± 4.3
2027 (II level)	85.4 83.8	84.6	1.3	

Limits of the method Limit of quantification: 0.05 mg/kg Limit of detection: 0.014 mg/kg Linearity range: from 2.5 to 100 ng/mL (corresponding to 0.014 - 0.56 mg/kg in undiluted samples) $r \geq 0.998$ Method validation range: 0.05 – 2027 mg/kg $_{DW}$

DW = Dry Weight

A 2.1.2.7.5 HPLC - LC-MS/MS (in sucrose solutions and in water)

A 2.1.2.7.5.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/05
Report	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Chronic Oral Toxicity Test Analytical Phase: Validation of an Analytical method and determination of content of prothioconazole in the feeding solutions of honey bees new born workers and in the water stock solution (OECD 245), Mautino G., 2023, Study Code: 1001.1F.SAG22; Test Site Code: 22293-01R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in the lowest concentration and in the highest concentration of the stock solutions and feeding solutions prepared in the biological phase of the study.
The analytical method for prothioconazole in sugar solution was fully validated in this study according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was extracted from the sucrose solution matrix with acetonitrile, after adding an opportune amount of water. After salts addition, the acetonitrile phase was separated from the aqueous phase.
The analytical method for prothioconazole in water matrix was fully validated in Renolab analytical phase 22293-03R according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021 and confirmed in the present study by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was determined in water stock solutions after sample dilution with acetonitrile.
For both matrices, the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Equipment

- Standard laboratory glassware and equipment
- Analytical electronical balance with 0.1 mg accuracy, AT 261, METTLER TOLEDO
- Centrifuge Megafuge 16, HERAEUS
- Automatic pipette 2-20 µL, Pipetman, GILSON

- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Water purification system Direct-Q 3UV, MILLIPORE
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCI-EX

Reagents and materials

- Acetonitrile for HPLC gradient grade, HONEYWELL ≥ 99.9%
- MilliQ Water
- Formic acid 98-100%, SIGMA ALDRICH
- QuEChERS extra packets, EN 15662 method, code 5982-7650, AGILENT
- Syringe PTFE filters 0.2 µm, 13 mm diameter, AGILENT
- Single use syringes

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile, 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: 4.8-5.5 minutes*
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

** two analytical columns of the same type were used during the study: slight differences in retention times are due to the different batches of the two columns stationary phase*

Analytical procedure for sucrose solution sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately weighed.
The homogenised sample (2.5 ± 0.05 g) was weighted (with 0.05 mg accuracy) into a 50 mL centrifuge tube; recovery samples were fortified at this point.
Then 7.5 mL of demineralised water were added and the sample was manually shaken for a few seconds to homogenise.
Then 10 mL of acetonitrile were added. In recovery trials, the 10 mL volume comprises the volume of the fortification solution added.
The tube was shaken vigorously by hand for 1 minute. After this step, the content of a sachet of QuEChERS EN 15662 pouch (Agilent code 5982-7650) was added to the sample.

The tube is shaken vigorously by hand for 1 minute and then centrifuged at 4000 rpm for five minutes.
The extract was filtered with PTFE filter, porosity 0.20 µm and finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.
Recovery samples at second level were diluted 1:10000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.
Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.

Analytical procedure for water sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately 1 mL is taken and brought to 5 mL with acetonitrile.
Recovery samples were prepared in water using the test item and processed.
Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
Recovery samples at second level were diluted 1:2000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The extract was finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.

Validation

Blank and selectivity
At least two independent analyses of the blank sample were performed for each matrix: no significant interference exceeding 20% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated sucrose solution sample used in fortification trials (< 20% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in sucrose solution and water matrices, without significant interferences above 20% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 10 - 1000 ng/mL corresponding to 0.04 – 4 mg/kg of prothioconazole in sucrose solution samples and 0.00005 to 0.005 g/L in water samples. The correlation coefficients of the weighed linear (1/x) multipoint external standard solvent calibration curves were found > 0.997 in all the analytical sequences performed.
The linearity range comprised the concentration range from 20% of the LOQ to 20% above the highest measured concentration.

The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;

yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.
The calculated prothioconazole concentration for all calibration control standards was within ± 20% of the nominal content.

Recoveries
The analytical method for sucrose solution matrix was validated by recovery trials while for matrix water recovery trials were performed to confirm the validation performed in study 22293-03R: a known quantity of the test item was added to the control sample and the percentage recovery calculated.
The recoveries for matrix sucrose solution and matrix water were performed by fortifying the untreated blank at two levels.
For matrix sucrose solution, the recoveries were performed by fortifying the untreated blank at two levels. The LOQ level was set at prothioconazole concentration of 0.8576 mg/kg, (lower than the minimum found prothioconazole content in samples), while the second level was at 20216 mg/kg (higher than the maximum expected concentration in the samples) in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; six replicated analyses were carried out for each fortification level.
The background content in the sucrose solution control sample used in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Sucrose solution matrix				
Fortification level (mg/kg)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.8576 (a) (LOQ)	90.0 95.1 93.3 94.2 93.7 78.8 85.5	90.1	6.6	91.7 ± 7.5
20216 (b) (II level)	92.0 98.7 83.9 84.9 106.5 93.4 93.4	93.3	8.4	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

For matrix water, the recoveries were performed by fortifying the untreated blank at two levels. The LOQ level was set at prothioconazole concentration of 0.00025 g/L, (lower than the minimum found prothioconazole content in samples), while the second level was at 5.0 g/L (higher than the maximum expected concentration in the samples) in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; three replicated analyses were carried out for each fortification level.
The background content in the water control sample used for the dilution of the test item in fortification experiments was not detectable.

In these recovery samples the prothioconazole content was determined as reported below.

Water matrix				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	96.2 92.9	94.6	2.5	92.9 ± 3.8
5.0 (b) (II level)	87.9 94.4	91.2	5.0	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

Accuracy
The accuracy of the analysis method for prothioconazole in sucrose solution matrix, defined as overall mean recovery ± relative standard deviation, is 91.7 ± 7.5.
The accuracy of the analysis method for prothioconazole in water matrix, defined as overall mean recovery ± relative standard deviation, is 92.9 ± 3.8.

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

Sucrose solution matrix		
Fortification level (mg/kg)	**RSD % (n = 7)**	**Overall RSD % (n = 14)**
0.8576 (a) (LOQ)	6.6	7.5
20216 (b) (II level)	7.4	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

Water matrix		
Fortification level (g/L)	**RSD % (n = 2)**	**Overall RSD % (n = 4)**
0.00025 (a) (LOQ)	2.5	3.8
5.0 (b) (II level)	5.0	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

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

Sucrose solution matrix		
Transition	**Matrix response over solvent response %**	**Matrix effects %**
344/326 (Target)	106	+ 6

344/125 (Qualifier)	92	- 8

No significant matrix effect (i.e. exceeding 20%) was found for prothioconazole in sucrose solution matrix, then solvent calibration standards were used for quantification of samples.
The matrix effect for prothioconazole in matrix water was found insignificant as well in Renolab study 22293-03R, then solvent calibration standards were used for quantification of samples.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in sucrose solution matrix the LOD is 0.04 mg/kg. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point.
For the analysis of prothioconazole in water matrix the LOD is 0.000015 g/L. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point.

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in sucrose solution matrix was assessed in this study at 0.8576 mg/kg (2.887 mg/kg referred as test item).
The LOQ for prothioconazole in water matrix was confirmed in this study at 0.00025 g/L (0.0.00085 g/L referred as test item).

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition in sucrose solution matrix and water matrix are reported in tables below.

Sucrose solution matrix				
Fortification level (mg/kg)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.8576 (a) (LOQ)	90.9 95.6 93.7 93.7 94.7 81.6 84.7	90.7	6.0	92.2 ± 6.9
20216 (b) (II level)	92.4 98.3 85.3 86.7 106.8 92.6 94.4	93.8	7.7	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

Water matrix				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	92.5 93.5	93.0	0.8	91.8 ± 3.4

5.0 (b) (II level)	87.1 93.9	90.5	5.3	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions

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

The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in sucrose solutions in the range 0.8576 – 20216 mg/kg (corresponding to 2.887 - 68044 mg/kg referred as test item) and in the range 0.00025 – 5.0 g/L (corresponding to 0.00085 - 17 mg/L referred as test item) for matrix water.

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

MATRIX SUCROSE SOLUTION: Prothioconazole (target transition 344/326)				
Fortification level (mg/kg)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.8576 (a) (LOQ)	90.0 95.1 93.3 94.2 93.7 78.8 85.5	90.1	6.6	91.7 ± 7.5
20216 (b) (II level)	92.0 98.7 83.9 84.9 106.5 93.4 93.4	93.3	8.4	
Limits of the method Limit of quantification: 0.8576 mg/kg Limit of detection: 0.04 mg/kg Linearity range: from 10 to 1000 ng/mL (corresponding to 0.04 - 4 mg/kg in undiluted samples) r ≥ 0.997 Method validation range: 0.8576 – 20216 mg/kg				

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

MATRIX WATER: Prothioconazole (target transition 344/326)

Fortification level (g/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.00025 (a) (LOQ)	96.2 92.9	94.6	2.5	92.9 ± 3.8
5.0 (b) (II level)	87.9 94.4	91.2	5.0	
Limits of the method Limit of quantification: 0.00025 g/L Limit of detection: 0.00005 g/L Linearity range: from 10 to 1000 ng/mL (corresponding to 0.00005 - 0.005 g/L in undiluted samples) $r \geq 0.997$ Method validation range: 0.00025 – 5.0 g/L				

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

A 2.1.2.7.6 HPLC - LC-MS/MS (in soil)

A 2.1.2.7.6.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/06
Report	Collembolan *Folsomia candida* reproduction test in soil with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of content of PROTHIOCONAZOLE in soil samples (OECD 232), Mautino G., 2023, Study Code: 1143.1F.SAG22; Test Site Code: 22293-05R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in soil samples collected during the biological phase of the study.
The analytical method for prothioconazole in soil was fully validated in Renolab analytical phase 22293-02R (Sagea Study code 1139.1F.SAG22) according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
In the current study the analytical method was reconfirmed in soil by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was extracted from soil samples with methanol and acidified water mixtures; the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Equipment

- Standard laboratory glassware and equipment

- Analytical electronical balance with 0.1 mg accuracy, AT 261, METTLER TOLEDO
- Technical electronic balance with 0.01 g accuracy PS 2100.R2, RADWAG
- Automatic pipette 2-20 µL, Pipetman, GILSON
- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Ultrasonic bath 2210, BRANSON
- Horizontal shaker with heating bath, H20S, LAUDA
- Centrifuge Megafuge 16, HERAEUS
- Water purification system Direct-Q 3UV, MILLIPORE
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCI-EX
- Thermostatic oven, model 11120-VF, MPM Instruments

Reagents and materials

- MilliQ Water from Direct-Q 3UV MILLIPORE
- Acetonitrile for UHPLC-MS, CARLO ERBA
- Methanol for UHPLC-MS, CARLO ERBA
- Formic acid ≥ 95%, SIGMA ALDRICH
- Acetic acid glacial ≥ 99.9%, CARLO ERBA
- Syringe PTFE filters 0.22 µm, 13 mm diameter, VWR
- Single use syringes
- 1 % acetic acid in water
- Methanol / 1% acetic acid in water 80/20 solvent mixture
- Methanol / 1% acetic acid in water 50/50 solvent mixture
- Eluent A: Water (milliQ), 0.1% formic acid
- Eluent B: Acetonitrile (UHPLC-MS), 0.1% formic acid

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile (UHPLC-MS), 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: ~5.5 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for soil samples analysis

The sample was let to warm up to room temperature.
The homogenised sample (6.00 ± 0.05 g) was weighted into a 50 mL centrifuge tube, recovery samples were fortified at this point.
Then 10 mL of methanol were added, the sample was sonicated for 5 minutes, manually shaken for a few seconds to homogenise, and shaken for 30 minutes with a horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer collected into a centrifuge tube.
The soil is further extracted with 8 mL of a mix methanol/1% acetic acid in water 80/20, then manually shaken for a few seconds to homogenise and shaken for 5 minutes by horizontal shaker. The sample was then centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
The soil is extracted a third time with 7 mL of a mix methanol/1% acetic acid in water 50/50, then manually shaken for a few seconds to homogenise and shaken for 5 minutes with a horizontal shaker.
Finally, the sample was centrifuged at 4000 rpm for five minutes and the supernatant layer was combined to the previous extract in the centrifuge tube.
All the extracts, combined in the centrifuge tube, were brought to a final volume of 25 mL with 1% acetic acid in water, then an aliquot of the extract was filtered on 0.22 µm PTFE membrane into a vial for the analysis.
Samples with analyte concentration exceeding 80 ng/mL were opportunely diluted with blank untreated extract in order to fall within ± 20% of the calibration range.
Recovery samples at second level were diluted 1:10000 with blank untreated extract in order to obtain final extract concentrations falling within ± 20 % of the calibration range.
The samples were analysed by high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS).
Quantification was conducted using an external standard calibration curve obtained by linear regression of matrix matched calibration standards injected throughout the run in the range 2.5 – 100 ng/mL.

Validation

Blank and selectivity
Four independent analyses of the blank sample were performed: no significant interference exceeding 30% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated soil sample used in fortification trials (< 30% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in soil matrix, without significant interferences above 30% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 2.5 - 100 ng/mL corresponding to 0.014 to 0.56 mg/kg of prothioconazole in dry soil sample. The correlation coefficient of the weighed linear (1/x) multipoint external matrix matched standard calibration curves was found ≥ 0.995 for both ion transitions in all the analytical sequences performed.
The linearity range comprised the concentration range from 30% of the LOQ to at least 20% above the highest measured concentration.

The suitability of the calibration lines was assessed using the residuals di that describes the vertical dis-

tance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;

yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

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

Recoveries

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

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

The LOQ level was set at prothioconazole concentration of 0.05 mg/kg dry weight (lower than the minimum found prothioconazole content in samples, while the second level was at 2015 mg/kg dry weight (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.

The background content in the control sample used for fortification experiments was not detectable. In these recovery samples the prothioconazole content was determined as reported below.

Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	88.8 93.5	91.2	3.6	84.8 ± 9.9
2015 (II level)	82.9 74.0	78.5	8.0	

DW = Dry Weight

For each fortification level the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy

The accuracy of the analysis method for prothioconazole in soil, defined as mean recovery ± relative standard deviation, is 84.8 ± 9.9.

Repeatability

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

Fortification level (mg/kg DW)	**RSD % (n = 2)**	**Overall RSD % (n = 4)**
0.05 (LOQ)	3.6	9.9
2015 (II level)	8.0	

DW = Dry Weight

Matrix Effect

To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of prothioconazole at 10 ng/mL (nearest to the nominal concentration for the LOQ level) was compared to prothioconazole in solvent at the same con-

centration. The results are summarized in the following table.

Transition	Matrix response over solvent response %	Matrix effects %
344/326 (Target)	76	- 24
344/125 (Qualifier)	76	- 24

Matrix effects for prothioconazole in soil matrix were found significant (> 20%) for both acquired transitions, then matrix matched calibration standards were used in the quantification of samples.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in soil the LOD is 0.014 mg/kg (referred to dry soil). This value, calculated from the prothioconazole concentration corresponding to the lowest calibration point, is below 30% of LOQ.

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in soil was assessed in this study at 0.05 mg/kg (referred to dry soil).

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg $_{DW}$)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.05 (LOQ)	93.5 95.8	94.7	1.7	86.3 ± 12.5
2015 (II level)	83.6 72.1	77.9	10.4	

DW = Dry Weight

Also, for the confirmatory transition, the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Stability of final extracts and reference item solutions
During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.
The final extracts were analysed within 24 hours form extraction. Moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.1.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable,

accurate and precise procedure for the determination of prothioconazole active ingredient in soil samples in the range 0.05 – 2015 mg/kg dry weight.
As the analysis of the samples was performed concurrently with the recovery tests, the reliability of the found values was demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	88.8 93.5	91.2	3.6	84.8 ± 9.9
2015 (II level)	82.9 74.0	78.5	8.0	
Limits of the method Limit of quantification: 0.05 mg/kg Limit of detection: 0.014 mg/kg Linearity range: from 2.5 to 100 ng/mL (corresponding to 0.014 - 0.56 mg/kg in undiluted samples) $r \geq 0.995$ Method validation range: 0.05 – 2015 mg/kg DW				

DW = Dry Weight

A 2.1.2.7.7 HPLC - LC-MS/MS (in ISO standard water)

A 2.1.2.7.7.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/07
Report	Daphnia sp. Acute Immobilization Test (*Daphnia magna*) with PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) Analytical Phase: Determination of the content of PROTHIOCONAZOLE in water medium samples (OECD 202), Artusio M., 2023, Study Code: 1136.1F.SAG22; Test Site Code: 22293-06R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in water medium exposure concentrations of Daphnia magna collected during the biological phase of the study.
The analytical method for prothioconazole was fully validated in water in Renolab analytical phase 22293-03R (Sagea Study code 1002.1F.SAG22) according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
In the current study the analytical method was reconfirmed in ISO standard water by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).

The water medium samples were analysed after dilution with acetonitrile. The final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LCMS/MS).

Equipment

- Standard laboratory glassware and equipment
- Analytical electronic balance with 0.1 mg accuracy, model AT 261, METTLER TOLEDO
- Automatic pipette FINNPIPETTE F2 100-1000 µL, THERMO SCIENTIFIC
- Automatic pipette FINNPIPETTE F2 20-200 µL, THERMO SCIENTIFIC
- Ultrasonic bath, model 2210, Branson
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCIEX
- System for pure and ultrapure water production, Direct-Q 3UV, Millipore

Reagents and materials

- Acetonitrile for UHPLC-MS, CARLO ERBA
- MilliQ Water produced by Direct-Q 3UV, Millipore
- Formic acid ≥ 95%, SIGMA ALDRICH
- Syringe PTFE filters 0.2 µm, 13 mm diameter, AGILENT
- Single use syringes
- Solvent mix: acetonitrile/water 50:50 v/v

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile (UHPLC-MS), 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 20 µL
- Retention time: Prothioconazole: ~5.5 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately 5 mL are transferred into a 10-mL volumetric flask; then the sample was brought to 10 mL with acetonitrile.

Samples with analyte concentration exceeding 160 ng/mL were opportunely diluted with blank untreated extract in order to fall within ± 20% of the calibration range.
Recovery samples at second level were diluted 1:10 with blank untreated extract in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The extract was finally filtered by 0.22 µm PTFE membrane and analysed by HPLC-MS/MS.
Quantification was performed using matrix matched calibration standards in the concentration range 2.5 - 200 ng/mL.

Validation

Blank and selectivity
The untreated ISO standard water was analysed in duplicate and prothioconazole was found not detectable. No significant interference (< 30% of LOQ) from the sample matrix were found.
Therefore, prothioconazole can be regarded as not detectable in the untreated water ISO standard water sample (< 30% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in ISO standard water, without significant interferences above 30% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 2.5 - 200 ng/mL corresponding to 0.005 – 0.4 mg/L of prothioconazole in water samples. The correlation coefficients of the weighed linear (1/x) multipoint external standard solvent calibration curves were found ≥ 0.995 in all the analytical sequences performed.
The linearity range comprised the concentration range from 30% of the LOQ to 20% above the highest measured concentration.

The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.
The calculated prothioconazole concentration for all calibration control standards was within ± 20% of the nominal content.

Recoveries
Procedural recoveries were performed by fortifying the untreated ISO standard water at two levels: LOQ and 2nd level.
The LOQ level was verified by three replicated recoveries at prothioconazole concentration of 0.020 mg/L, (lower than the minimum found prothioconazole concentration in samples), while the second level was verified by three replicated recoveries at 3.1 mg/L (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of prothioconazole concentra-

tions in the analytical samples.
The background content in the control sample used in fortification experiments was not detectable.
In these recovery samples the prothioconazole content in was determined as reported below.

Fortification level (mg/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.020 (a) (LOQ)	73.9 71.9 77.4	74.4	3.7	75.3 ± 3.1
3.1 (b) (II level)	78.3 75.5 74.5	76.1	2.6	

(a) 0.068 mg/L as test item
(b) 10.4 mg/L as test item

For each fortification level the mean recovery was in the range 70-120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Matrix Effect
To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of prothioconazole at 10 ng/mL (nominal concentration for the LOQ level) was compared to prothioconazole in solvent at the same concentration.
The results are summarized in the following table.

Transition	Matrix response over solvent response %	Matrix effects %
344/326 (Target)	118	+ 18
344/125 (Qualifier)	123	+ 23

Matrix effect for prothioconazole in ISO standard water was found significant (> 20%) for one acquired transition (qualifier), then matrix matched calibration standards were used for quantification of samples.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value: it is calculated from the prothioconazole concentration corresponding to the lowest calibration point.
For the analysis of prothioconazole in ISO standard water the LOD is 0.005 mg/L (< 30% of LOQ).

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in ISO standard water was confirmed in this study at 0.020 mg/L.

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional qualifier transition.
The recovery data and the precision data for the additional transition are reported respectively in following table.

Fortification level (mg/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %

0.020 (a) (LOQ)	70.0 69.9 74.9	71.6	4.0	73.9 ± 4.6
3.1 (b) (II level)	78.5 75.4 74.9	76.3	2.6	

(a) 0.068 mg/L as test item
(b) 10.4 mg/L as test item

Also, for the confirmatory transition, the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Stability of final extracts and reference item solutions

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

The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.1.

Conclusion

The data presented in this report confirm that the analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in ISO standard water in the range 0.020 – 3.1 mg/L.

Prothioconazole in ISO standard water (target transition 344/326)				
Fortification level (mg/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.020 (a) (LOQ)	73.9 71.9 77.4	74.4	3.7	75.3 ± 3.1
3.1 (b) (II level)	78.3 75.5 74.5	76.1	2.6	
Limits of the method Limit of quantification: 0.020 mg/L Limit of detection: 0.005 mg/L Linearity range: from 2.5 to 200 ng/mL (corresponding to 0.005 – 0.4 mg/L in undiluted samples) r ≥ 0.995 Method validation range: 0.020 – 3.1 mg/L				

(a) 0.068 mg/L as test item
(b) 10.4 mg/L as test item

A 2.1.2.7.8 HPLC - LC-MS/MS (in sucrose solutions and in water)

A 2.1.2.7.8.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference: KCP 5.1.2/08

Report	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Bumblebee (*Bombus terrestris* L.) in the laboratory – Acute Oral and Contact Toxicity Test Analytical Phase: Determination of content of prothioconazole in the feeding solutions and in the water contact solution for bumblebees (OECD 246 and 247), Mautino G., 2023, Study Code: 1138.1F.SAG22; Test Site Code: 22293-07R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in the lowest concentration and in the highest concentration of the sucrose feeding solutions and in the contact water solution prepared in the biological phase of the study.

The analytical method for prothioconazole in sugar solution was fully validated in Renolab analytical phase 22293-01R according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021 and confirmed in the present study by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was extracted from the sucrose solution matrix with acetonitrile, after adding an opportune amount of water. After salts addition, the acetonitrile phase was separated from the aqueous phase.

The analytical method for prothioconazole in water matrix was fully validated in Renolab analytical phase 22293-03R according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021 and confirmed in the present study by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was determined in water solutions after sample dilution with acetonitrile.

For both matrices, the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).
Two transitions m/z 344 > 326 and m/z 344 > 125 were acquired: the first transition for quantification purpose, the second transition for confirmation purpose.

Equipment

- Standard laboratory glassware and equipment
- Water purification system Direct-Q 3UV, MILLIPORE
- Analytical electronical balance with 0.1 mg readability AT 261, METTLER TOLEDO
- Technical electronic balance accurate to 0.01 g, model PS 2100.R2, RADWAG
- Centrifuge Megafuge 16, HERAEUS
- Automatic pipette 2-20 µL, Pipetman P20, GILSON
- Automatic pipette 20-200 µL, Finpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, Finpipette F2, THERMO SCIENTIFIC
- Ultrasonic bath, model 2210, Branson
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCIEX

Reagents and materials

- Acetonitrile for HPLC gradient grade, HONEYWELL ≥ 99.9%
- MilliQ Water produced by Direct-Q 3UV, MILLIPORE
- Formic acid ≥ 95%, SIGMA ALDRICH

- QuEChERS extractive kit, EN 15662 method, Sharlau
- Syringe PTFE filters 0.22 µm, 13 mm diameter, VWR
- Single use syringes

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile, 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: ~ 5.7 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for sucrose solution sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately weighed.
The homogenised sample (2.5 ± 0.05 g) was weighted (with 0.05 mg accuracy) into a 50 mL centrifuge tube; recovery samples were fortified at this point.
Then 7.5 mL of demineralised water were added and the sample was manually shaken for a few seconds to homogenise.
Then 10 mL of acetonitrile were added. In recovery trials, the 10 mL volume comprises the volume of the fortification solution added.
The tube was shaken vigorously by hand for 1 minute. After this step, the content of a sachet of QuEChERS EN 15662 pouch (Agilent code 5982-7650) was added to the sample.
The tube is shaken vigorously by hand for 1 minute and then centrifuged at 4000 rpm for five minutes.
The extract was filtered with PTFE filter, porosity 0.20 µm and finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.
Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
Recovery samples at second level were diluted 1:10000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.

Analytical procedure for water sample extracts preparation

The sample was let to warm up to room temperature, then sonicated for 5 minutes, vigorously shaken by hand for a further minute and immediately 1 mL is taken and brought to 5 mL with acetonitrile.
Recovery samples were prepared in water using the test item and processed.

Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
Recovery samples at second level were diluted 1:100000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The extract was finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.

Validation

Blank and selectivity
Two independent analyses of the blank sample were performed for each matrix: no significant interference exceeding 20% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated sucrose solution sample and in the water used in fortification trials (< 20% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in sucrose solution and water matrices, without significant interferences above 20% of LOQ.

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum (product ion chromatogram) of the analyte was acquired in Renolab analytical phase 22293-01R in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 10 - 1000 ng/mL corresponding to 0.04 – 4 mg/kg of prothioconazole in sucrose solution samples and 0.00005 to 0.005 g/L in water samples.
The correlation coefficients of the weighed linear (1/x) multipoint external standard solvent calibration curves were found > 0.998 in all the analytical sequences performed.
The linearity range comprised the concentration range from 20% of the LOQ to 20% above the highest measured concentration.
The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;

yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.
See Appendix 1 for the calibration curves and residuals plots obtained during the analysis.
The calculated prothioconazole concentration for all calibration control standards was within ± 20% of the nominal content.

Recoveries
The analytical method for sucrose solution matrix was validated by recovery trials in Renolab analytical phase 22293-01R while for matrix water the validation was performed in Renolab analytical phase 22293-03R. In the present study procedural recoveries were performed for both matrices to confirm the

validations: known quantities of the test item were added to the control samples and the percentage recovery calculated.
The recoveries for matrix sucrose solution and matrix water were performed by fortifying the untreated blank at two levels.
For matrix sucrose solution, procedural recoveries were performed by fortifying the untreated blank at two levels. The LOQ level was set at prothioconazole concentration of 0.8576 mg/kg, (lower than the minimum expected prothioconazole content in the treated samples), while the second level was at 20216 mg/kg (higher than the maximum expected prothioconazole content in the treated samples) in order to cover with the method validation, the prothioconazole concentrations in the analytical samples; two replicated analyses were carried out for each fortification level.
The background content in the sucrose solution control sample used in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Sucrose solution matrix				
Fortification level (mg/kg)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.8576 (a) (LOQ)	71.2 81.5	76.4	9.5	76.4 ± 5.5
20216 (b) (II level)	76.2 76.5	76.4	0.3	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

For each fortification level the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20%, in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

For matrix water, the recoveries were performed by fortifying the untreated blank at two levels. The LOQ level was set at prothioconazole concentration of 0.000245 g/L, (lower than the expected prothioconazole content in the treated sample), while the second level was at 61.3 g/L (higher than the expected prothioconazole content in the treated sample) in order to cover with the method the prothioconazole concentration in the analytical sample; two replicated analysis were carried out for LOQ level and three for the second level.
The background content in the water control sample used for the dilution of the test item in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Water matrix				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.000245 (a) (LOQ)	84.6 80.5	82.6	3.5	85.2 ± 3.9
61.3 (b) (II level)	84.1 87.4 89.2	86.9	3.0	

(a) 0.000825 g/L as test item
(b) 206 g/L as test item

Accuracy
The accuracy of the analysis method for prothioconazole in sucrose solution matrix, defined as overall mean recovery ± relative standard deviation, is 76.4 ± 5.5.
The accuracy of the analysis method for prothioconazole in water matrix, defined as overall mean recovery ± relative standard deviation, is 85.2 ± 3.9.

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

Sucrose solution matrix		
Fortification level (mg/kg)	**RSD % (n = 2)**	**Overall RSD % (n = 4)**
0.8576 (a) (LOQ)	9.5	5.5
20216 (b) (II level)	0.3	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

Water matrix		
Fortification level (g/L)	**RSD %**	**Overall RSD % (n = 5)**
0.000245 (a) (LOQ)	3.5 (n = 2)	3.9
61.3 (b) (II level)	3.0 (n = 3)	

(a) 0.000825 g/L as test item
(b) 206 g/L as test item

Matrix Effect
No significant matrix effect (i.e. exceeding 20%) was found for prothioconazole for sucrose solution matrix in Renolab analytical phase 22293-01R and for water matrix in Renolab analytical phase 22293-03R, then solvent calibration standards were used for quantification of samples.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in sucrose solution matrix the LOD is 0.04 mg/kg. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point.
For the analysis of prothioconazole in water matrix the LOD is 0.000015 g/L. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point.

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in sucrose solution matrix was confirmed in this study at 0.8576 mg/kg (2.887 mg/kg referred as test item).
The LOQ for prothioconazole in water matrix was confirmed in this study at 0.000245 g/L (0.0.000825 g/L referred as test item).

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition in sucrose solution matrix and water matrix are reported in tables below.

Sucrose solution matrix

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.8576 (a) (LOQ)	71.5 81.5	76.5	9.2	76.0 ± 5.4
20216 (b) (II level)	75.6 75.5	75.6	0.1	

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

Water matrix				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.000245 (a) (LOQ)	84.3 79.9	82.1	3.8	84.3 ± 3.3
61.3 (b) (II level)	84.0 86.8 86.6	85.8	1.8	

(a) 0.000825 g/L as test item
(b) 206 g/L as test item

Also, for the confirmatory transition, the mean recovery was in the range 70 – 120% and the precision (RSD, relative standard deviation) ≤ 20% at each fortification level, in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions

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

The prothioconazole reference item stock solution was proven to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab study 22293-03R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in sucrose solutions in the range 0.8576 – 20216 mg/kg (corresponding to 2.887 - 68044 mg/kg referred as test item) and in the range 0.000245 – 61.3 g/L (corresponding to 0.000825 - 206 g/L referred as test item) for matrix water.

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

MATRIX SUCROSE SOLUTION: Prothioconazole (target transition 344/326)				
Fortification level (mg/kg)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.8576 (a) (LOQ)	71.2 81.5	76.4	9.5	76.4 ± 5.5
20216 (b) (II level)	76.2 76.5	76.4	0.3	
Limits of the method Limit of quantification: 0.8576 mg/kg Limit of detection: 0.04 mg/kg Linearity range: from 10 to 1000 ng/mL				

(corresponding to 0.04 - 4 mg/kg in undiluted samples) r ≥ 0.999 Method validation range: 0.8576 – 20216 mg/kg

(a) 2.887 mg/kg as test item
(b) 68044 mg/kg as test item

MATRIX WATER: Prothioconazole (target transition 344/326)				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.000245 (a) (LOQ)	84.6 80.5	82.6	3.5	85.2 ± 3.9
61.3 (b) (II level)	84.1 87.4 89.2	86.9	3.0	
Limits of the method Limit of quantification: 0.000245 g/L Limit of detection: 0.00005 g/L Linearity range: from 10 to 1000 ng/mL (corresponding to 0.00005 - 0.005 g/L in undiluted samples) r ≥ 0.999 Method validation range: 0.000245 – 61.3 g/L				

(a) 0.000825 g/L as test item
(b) 206 g/L as test item

A 2.1.2.7.9 HPLC - LC-MS/MS (in water)

A 2.1.2.7.9.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/09
Report	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on terrestrial Non-target plants – Seedling Emergence and Seedling growth Analytical Phase: Determination of the content of PROTHIOCONAZOLE in the water spray solutions (OECD 208), Mautino G., 2023, Study Code: Study Code: 1140.1F.SAG22; Test Site Code: 22293-08R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method for prothioconazole in water was fully validated in Renolab analytical phase 22293-03R (Sagea study code 1002.1F.SAG22) according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).
In the current study the analytical method was reconfirmed by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, matrix effects, limit of detection (LOD) and limit of quantification (LOQ).

Prothioconazole was determined in water stock solution after sample dilution with acetonitrile and the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).
Two transitions m/z 344 > 326 and m/z 344 > 125 were acquired: the first transition for quantification purpose, the second transition for confirmation purpose.

Equipment

- Standard laboratory glassware and equipment
- Water purification system Direct-Q 3UV, MILLIPORE
- Analytical electronical balance with 0.1 mg readability AT 261, METTLER TOLEDO
- Automatic pipette 2-20 µL, Pipetman P20, GILSON
- Automatic pipette 20-200 µL, Finnpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, DV 1000, DISCOVERY COMFORT
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCI-EX

Reagents and materials

- Acetonitrile for UHPLC-MS gradient grade, CARLO ERBA
- MilliQ Water produced in lab by Direct-Q 3UV, MILLIPORE
- Formic acid ≥95%, SIGMA ALDRICH
- Syringe PTFE filters 0.2 µm, J.T. BAKER
- Single use syringes

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile, 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 10%
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: 5.6 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for sample extracts preparation

The sample was let to warm up to room temperature, vigorously shaken by hand for a minute and immediately 1 mL is taken and brought to 5 mL with acetonitrile.
Recovery samples were prepared in water using the test item and processed.

Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
Recovery samples at second level for matrix water were diluted 1:2000 with acetonitrile in order to obtain final extract concentrations falling within ± 20% of the calibration range.
The extract was finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.

Validation

Blank and selectivity
Two independent analyses of the blank sample were performed: no significant interference exceeding 10 % of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated water sample used in fortification trials (< 10% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of prothioconazole in water solution matrix, without significant interferences above 10% of LOQ (taking into account the operative dilution of LOQ level).

Specificity
Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision
The linearity range for prothioconazole was found between 10 - 1000 ng/mL corresponding to the range from 0.00005 to 0.005 g/L of prothioconazole in water samples. The correlation coefficient of the weighed linear (1/x) multipoint external standard solvent calibration curve was found ≥ 0.999 in the analytical sequence performed.
The linearity range comprised the concentration range from 10% of the LOQ (taking into account the operative dilution of LOQ level) to at least 20 % above the highest measured concentration.

The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.

Recoveries
The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.
Procedural recoveries were performed by fortifying the untreated blank at two levels.
The LOQ level was set at prothioconazole concentration of 0.00025 g/L (0.00085 g/L as test item), lower than the minimum found prothioconazole content in samples, while the second level was at 5.05 g/L (17 g/L as test item), higher than the maximum expected concentration in the samples, in order to cover with

the method validation all the range of prothioconazole concentrations in the analytical samples. Two replicated analyses were carried out for each fortification level.
The background content in the control sample used for dilution of test item in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Fortification level (g/L)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.00025 (a) (LOQ)	103.0 90.9	97.0	8.8	92.1 ± 8.4
5.05 (b) (II level)	89.2 85.1	87.2	3.3	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy
The accuracy of the analysis method for prothioconazole in water, defined as mean recovery ± relative standard deviation, is 92.1 ± 8.4.

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

Fortification level (g/L)	RSD % (n = 2)	Overall RSD % (n = 4)
0.00025 (a) (LOQ)	8.8	8.4
5.05 (b) (II level)	3.3	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

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

Transition	Matrix response over solvent response %	Matrix effects %
344/326 (Target)	98	- 2
344/125 (Qualifier)	103	+ 3

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

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in water the LOD is 0.00005 g/L. This value was calculated from the

prothioconazole concentration corresponding to the lowest calibration point (undiluted sample).

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in water was assessed in this study at 0.00025 g/L.

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	101.5 89.3	95.4	9.0	91.8 ± 7.3
5.05 (b) (II level)	90.1 86.1	88.1	3.2	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions
The final extracts were analysed within 24 hours form extraction; moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The stability of prothioconazole reference item stock solution was verified to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab analytical phase 22293-03R (Sagea study code 1102.1F.SAG22): the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in water samples in the range 0.00025 – 5.05 g/L (corresponding to 0.00085 – 17 g/L as test item).
As the analysis of the samples was performed during the recovery tests, the reliability of the found values is demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	103.0 90.9	97.0	8.8	92.1 ± 8.4
5.05 (b) (II level)	89.2 85.1	87.2	3.3	
Limits of the method Limit of quantification: 0.00025 g/L Limit of detection: 0.00005 g/L Linearity range: from 10 to 1000 ng/mL (corresponding to 0.00005 - 0.005 g/L in undiluted samples)				

$r \geq 0.999$ Method validation range: 0.00025 – 5.05 g/L

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

A 2.1.2.7.10 HPLC - LC-MS/MS (in water)

A 2.1.2.7.10.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference:	KCP 5.1.2/10
Report	Effects of PROTIOKONAZOL 300 EC (prothioconazole 300 g/L) on Terrestrial Plant Vegetative Vigour – OECD 227 Analytical Phase: Determination of the content of PROTHIOCONAZOLE in the water spray solution, Mautino G., 2023, Study Code: 1141.1F.SAG22; Test Site Code: 22293-09R
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The content of prothioconazole active ingredient was determined in the highest concentration of the test solution prepared in the biological phase of the study.
The analytical method for prothioconazole in water was fully validated in Renolab analytical phase 22293-03R (Sagea study code 1002.1F.SAG22) according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD). In the current study the analytical method was reconfirmed by calibration (linearity), selectivity, specificity, blank samples analysis, procedural recoveries, matrix effects, limit of detection (LOD) and limit of quantification (LOQ).
Prothioconazole was determined in water stock solution after sample dilution with acetonitrile and the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).
Two transitions m/z 344 > 326 and m/z 344 > 125 were acquired: the first transition for quantification purpose, the second transition for confirmation purpose.

Equipment

- Standard laboratory glassware and equipment
- Water purification system Direct-Q 3UV, MILLIPORE
- Analytical electronical balance with 0.1 mg readability AT 261, METTLER TOLEDO
- Automatic pipette 2-20 µL, Pipetman P20, GILSON
- Automatic pipette 20-200 µL, Finnpipette F2, THERMO SCIENTIFIC
- Automatic pipette 100-1000 µL, DV 1000, DISCOVERY COMFORT
- HPLC column Poroshell 120 SB C18, 2.1 x 100 mm, 2.7 µm, AGILENT
- LC/MS/MS System HPLC Series 1200 AGILENT and MS/MS Spectrometer API 3200 AB SCIEX

Reagents and materials

- Acetonitrile for UHPLC-MS gradient grade, CARLO ERBA
- MilliQ Water produced in lab by Direct-Q 3UV, MILLIPORE
- Formic acid ≥95%, SIGMA ALDRICH
- Syringe PTFE filters 0.2 µm, J.T. BAKER
- Single use syringes

Instrumental parameters LC-MS/MS

- LC System: HPLC Series 1200 Agilent
- MS/MS detector: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- System Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Acetonitrile, 0.1% formic acid
- Pump Gradient: 0 min: A 90% - B 10%
  5 min: A 0% - B 100%
  10 min: A 0% - B 100%
  10.5 min: A 90% - B 10%
  14 min: A 90% - B 1 %
- Flow rate: 0.3 mL/min
- Column Temperature: 35°C
- Injection volume: 10 µL
- Retention time: Prothioconazole: 5.6 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
  Temperature (TEM): 500°C
  Curtain gas (CUR): 20 psi
  Collision gas (CAD): 5 psi
  Ion Spray Voltage (IS): 4500 V
  Gas 1: 45 psi
  Gas 2: 45 psi
  DP: 28
  EP: 2.4
- Transitions: PROTHIO-1_T (344/326) CE 5, CXP 5, dwell time 200 msec
  PROTHIO-2_Q (344/125): CE 53, CXP 2.4, dwell time 200 msec

Analytical procedure for sample extracts preparation

The sample was let to warm up to room temperature, vigorously shaken by hand for a minute and immediately 1 mL is taken and brought to 5 mL with acetonitrile.
Recovery samples were prepared in water using the test item and processed.
Sample extracts with analyte concentration exceeding 800 ng/mL were opportunely diluted with acetonitrile in order to fall within the ± 20% of the calibration range.
The extract was finally analysed by HPLC-MS/MS.
Quantification was performed using solvent calibration standards in the concentration range 10 -1000 ng/mL.

Validation

Blank and selectivity
Two independent analyses of the blank sample were performed: no significant interference exceeding 10% of the limit of quantification were found at the retention time of prothioconazole for both the monitored transitions.
Therefore, prothioconazole can be regarded as not detectable in untreated water sample used in fortification trials (< 10% of LOQ).
The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.
Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of

prothioconazole in water solution matrix, without significant interferences above 10% of LOQ (taking into account the operative dilution of LOQ level).

Specificity

Prothioconazole was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.
The mass spectrum of the analyte was acquired in the range 100-400 m/z.

Linearity and instrumental precision

The linearity range for prothioconazole was found between 10 - 1000 ng/mL corresponding to the range from 0.00005 to 0.005 g/L of prothioconazole in water samples. The correlation coefficient of the weighed linear (1/x) multipoint external standard solvent calibration curve was found ≥ 0.999 in the analytical sequence performed.
The linearity range comprised the concentration range from 10% of the LOQ (taking into account the operative dilution of LOQ level) to at least 20 % above the highest measured concentration.

The suitability of the calibration line was assessed using the residuals di that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i;
yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.
The linearity range comprised the concentration range of the samples ± 20%.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.
Procedural recoveries were performed by fortifying the untreated blank at two levels.
The LOQ level was set at prothioconazole concentration of 0.00025 g/L (0.00085 g/L as test item), lower than the minimum found prothioconazole content in samples, while the second level was at 5.05 g/L (17 g/L as test item), higher than the maximum expected concentration in the samples, in order to cover with the method validation all the range of prothioconazole concentrations in the analytical samples. Two replicated analyses were carried out for each fortification level.
The background content in the control sample used for dilution of test item in fortification experiments was not detectable.
In these recovery samples the prothioconazole content was determined as reported below.

Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	103.0 90.9	97.0	8.8	92.1 ± 8.4
5.05 (b) (II level)	89.2 85.1	87.2	3.3	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.1.

Accuracy

The accuracy of the analysis method for prothioconazole in water, defined as mean recovery ± relative standard deviation, is 92.1 ± 8.4.

Repeatability

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

Fortification level (g/L)	**RSD % (n = 2)**	**Overall RSD % (n = 4)**
0.00025 (a) (LOQ)	8.8	8.4
5.05 (b) (II level)	3.3	

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

Matrix Effect

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

Transition	**Matrix response over solvent response %**	**Matrix effects %**
344/326 (Target)	98	- 2
344/125 (Qualifier)	103	+ 3

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

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of prothioconazole in water the LOD is 0.00005 g/L. This value was calculated from the prothioconazole concentration corresponding to the lowest calibration point (undiluted sample).

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for prothioconazole in water was assessed in this study at 0.00025 g/L.

Confirmation

The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	101.5 89.3	95.4	9.0	91.8 ± 7.3
5.05 (b) (II level)	90.1 86.1	88.1	3.2	

(a) 0.00085 g/L as test item

(b) 17 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) at each fortification level are in compliance with the requirement of guideline SANTE/2020/12830, Rev.1.

Stability of final extracts and reference item solutions
The final extracts were analysed within 24 hours form extraction; moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The stability of prothioconazole reference item stock solution was verified to be stable for 36 days after preparation at ≤ - 18°C in the dark in Renolab analytical phase 22293-03R (Sagea study code 1102.1F.SAG22): the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18°C in the dark) did not differ by more than 10%.

Conclusion

The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of prothioconazole active ingredient in water samples in the range 0.00025 – 5.05 g/L (corresponding to 0.00085 – 17 g/L as test item).
As the analysis of the samples was performed during the recovery tests, the reliability of the found values is demonstrated.

Prothioconazole (target transition 344/326)				
Fortification level (g/L)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.00025 (a) (LOQ)	103.0 90.9	97.0	8.8	92.1 ± 8.4
5.05 (b) (II level)	89.2 85.1	87.2	3.3	
Limits of the method Limit of quantification: 0.00025 g/L Limit of detection: 0.00005 g/L Linearity range: from 10 to 1000 ng/mL (corresponding to 0.00005 - 0.005 g/L in undiluted samples) r ≥ 0.999 Method validation range: 0.00025 – 5.0 g/L				

(a) 0.00085 g/L as test item
(b) 17 g/L as test item

A 2.1.2.7.11 HPLC/MS-QQQ (in aqueous solutions)

A 2.1.2.7.11.1 Method validation

Comments of zRMS:	Method is accepted as pre-authorization method

Reference: KCP 5.1.2/11

Report: Validation of the analytical method for the determination of prothioconazole content in aqueous solutions coming from ecotoxicological tests with PROTIOKONAZOL 300 EC, Tediosi E., 2023, Study Code: CH – 0912-2022

STUDY PLAN AMENDMENT No.1
Validation of the analytical method for the determination of prothioconazole

	content in aqueous solutions coming from ecotoxicological tests with PROTIOKONAZOL 300 EC
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The aim of this study was to validate the analytical method for the determination of prothioconazole content in aqueous solutions that will come from the algal growth inhibition study on green algae *Raphidocelis subcapitata* (also known as *Pseudokirchneriella subcapitata*).
The analytical method for prothioconazole in aqueous solutions was fully validated in this study according to the guideline SANTE/2020/12830 rev.1, of 24 February 2021, by linearity, selectivity and specificity, confirmation, recovery, accuracy, precision, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).
Prothioconazole was determined in aqueous solutions by High Performance Liquid Chromatography with triple quadrupole-mass spectrometer (HPLC/MS-QQQ).

Equipment

- HPLC/MS-QQQ, Shimadzu Technologies, mod. 8050, equipped with binary pump, autosampler, f dcoupled with an ESI interfaced Triple Quadrupole Mass Detector, managed by LabSolution r software.443
- Analytical balance
- Freezer
- Refrigerator
- Aspirated Safety Storage Cabinet
- Water purification system
- Usual laboratory glassware
- Volumetric glassware class A

Reagents and materials

- Acetonitrile, HPLC grade
- Water, HPLC grade
- Formic acid
- Ammonium formate

Chromatographic conditions

- Column: HPLC column, Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d.; Internal code LCN 307 (or equivalent)
- Detector: MS Triple quadrupole (Scan in MRM mode)
- Column temperature: Not Controlled
- Eluent A: Water / formic acid 0.1% / ammonium formate 10 mM
- Eluent B: Acetonitrile
- Eluent flow: 0.7 mL/min
- Elution mode: Isocratic condition
- Mixture:

% A	% B
40	60

- Waste*

Command	Time (min)
Waste on*	4

- Volume of injection: 5 µL
- Retention time: Prothioconazole - Approximately 5.6 minutes
- Total analysis time: 12 minutes

Mass scan parameters

- Ion mode: ESI, positive polarity
- Scan type: MRM
- Interface temperature (°C): 300
- DL temperature (°C): 250
- Heat Block (°C): 300
- Dry gas flow (L/min): 10.0
- Nebulizing Gas Flow (L/min): 2.7
- Heating Gas Flow (L/min): 5.0
- Compound: Prothioconazole
- Precursor ion (m/z): 344.0
- Product ions (m/z)
  Quantifier 326.0
  Collision Energy: - 12
  Q1 Pre Bias - 18.0
  Q3 Pre Bias - 23.0
- Product ions (m/z)
  Qualifier 1: 189.0
  Collision Energy: - 20
  Q1 Pre Bias - 18.0
  Q3 Pre Bias – 19.0
- Product ions (m/z)
  Qualifier 2: 154.0
  Collision Energy: - 31
  Q1 Pre Bias - 18.0
  Q3 Pre Bias – 16.0
- Dwell time (msec) 200

** during the analysis the matrix eluting in the first minutes is sent to waste.*

Sample preparation

Aqueous solutions were prepared in Algal growth medium in accordance with the guideline OECD No. 201 (2011).
The samples were injected without any particular treatment, only eventual dilution in matrix.
After the analysis the samples were stored tightly closed at approximately 4°C.

Validation

Linearity/Calibration
The analytical calibration was performed by injecting, in duplicate determination, five matrix matched standard (MSS) solutions prepared in blank matrix (Algal growth medium).
Linear regression analysis was performed using the least squares method; the correlation coefficient was calculated using regression analysis.
To check linearity, five matrix-matched standard solutions were prepared as previously described in the experimental section and each solution was analysed by HPLC/MS/MS in duplicate.
The tested linearity range was 2.0 – 195.8 µg a.i./L, which corresponds to 6.7 – 659.0 µg test item/L as injected concentration and 6.7 µg test item/L – 131.8 mg test item/L, if injected samples are 200-fold diluted.
The range tested for the analyte was found to be linear (correlation coefficient $r \geq 0.99$).

	Parameter m (slope)	**Parameter q (intercept)**	**Parameter r (Correlation Coefficient)**	**R^2**
Prothioconazole in Algal growth medium ***quantifier transition m/z 344.0 → m/z 326.0***				
Conc. in µg/L	6756	-5354	0.99986	0.99973
Prothioconazole in Algal growth medium ***qualifier 1 transition m/z 344.0 → m/z 154.0***				

Conc. in µg/L	1659	-2115	0.99996	0.99993
Prothioconazole in Algal growth medium *qualifier 2 transition m/z 344.0 → m/z 189.0*				
Conc. in µg/L	1832	-2347	0.99984	0.99968

Selectivity and Specificity

The Prothioconazole determination was conducted by HPLC-MS/MS in MRM mode, monitoring three MS/MS ion mass transitions 344.0 → 326.0 for quantification, 344.0 → 154.0 and 344.0 → 189.0 for qualitative purpose (confirmation).

The SANTE/2020/12830, Rev.1 guideline requires that the specificity of instrumental response to the substance presence is demonstrated.

The Blank values (untreated samples) should not exceed 30% of the LOQ.

The specificity test was conducted injecting the following samples and comparing the signals to check for possible cross contaminations:

- Solvent wash (HPLC grade water)
- Blank matrix sample (Algal growth medium)
- Standard at lowest calibration level in matrix (MSS1)
- A fortified sample in matrix at lowest level (LOQ)

A comparison of the signal obtained for the different solutions shows that, following the operating conditions recommended in the analytical method, the response of blank matrix samples was comparable to the response of the fortified matrix samples at low level (LOQ).

Blank matrix samples response and LOQ response were compared and % Ratios (Blank vs LOQ) is reported in the following table.

Injected solutions	**% Blank vs LOQ**
Blank sample (Algal growth medium)	0%
A fortified Algal growth medium sample at lowest level (LOQ)	

In this way the ratio between blank matrix samples intensity and LOQ intensity (% Ratio BLK/LOQ) did not exceed 30% suggested by the guideline.

Recovery (Accuracy) and Repeatability (Precision)

The recovery and the repeatability (as precision, % RSD) of the analytical method were determined using freshly fortified control samples of Algal growth medium.

Fortification levels were chosen at the LOQ and at the higher concentration tested in ecotoxicological studies.

The samples were fortified at following nominal concentrations:

- Low level: 25 µg test item/L (7.4 µg Prothioconazole / L);
- High level: 100 mg test item/L, in algal growth medium (29.71 mg Prothioconazole / L);

Fortified samples were quantified using the equation of the calibration curve, with the standard solutions injected in the same run with the samples (bracketing calibration): five matrix-matched standard solutions (from MSS1 to MSS5) were prepared in matrix and they were injected before and after the fortification levels (i.e., two series of injections).

Bracketing calibration allowed a more precise quantification, since intensity obtained from the analysis of fortified samples were compared to intensity of standard solutions analysed immediately before and after samples, avoiding problems due to variation of instrumental response during analysis.

Recovery and Repeatability: analysis of the Algal growth medium fortified samples at the low level (LOQ)

Sample ID	**Area**	**Cs [(1)] (µg/L)**	**Dilution**	**found (µg/L)**	**added (µg/L)**	**Recovery %**
CNT A1_Algal growth medium	0	0.0	1	n.d.	0.0	-

CNT A2_Algal growth medium	0	0.0	1	n.d.	0.0	-
Spike Low A_Algal growth medium	54499	8.9	1	8.9	7.6	116.9
Spike Low B_Algal growth medium	55092	8.9	1	8.9	7.6	118.1
Spike Low C_Algal growth medium	50965	8.3	1	8.3	7.6	110.0
Spike Low D_Algal growth medium	50722	8.3	1	8.3	7.6	109.6
Spike Low E_Algal growth medium	42785	7.1	1	7.1	7.6	94.1
			Mean value	8.31	7.6	109.7
			Standard Deviation (SD)	0.73		9.6
			Relative Standard Deviation (RSD%)	**9%**		**9%**

(1) Quantification with the linear calibration curve

Recovery and Repeatability: analysis of the Algal growth medium fortified samples at the high level (Spike High)

Sample ID	Area	Cs (1) (µg/L)	Dilution	found (µg/L)	added (µg/L)	Recovery %
CNT B1_Algal growth medium	0	0.0	200	n.d.	0.00	-
CNT B2_Algal growth medium	0	0.0	200	n.d.	0.00	-
Spike High A_Algal growth medium	1030085	156.7	200	31.35	30.30	103.4
Spike High B_Algal growth medium	1022068	155.5	200	31.11	30.30	102.6
Spike High C_Algal growth medium	1053039	160.2	200	32.05	30.30	105.7
Spike High D_Algal growth medium	1023725	155.8	200	31.16	30.30	102.8
Spike High E_Algal growth medium	993917	151.3	200	30.25	30.30	99.8
			Mean value	31.18		102.9
			Standard Deviation (SD)	0.64		2.1
			Relative Standard Deviation (RSD%)	**2%**		**2%**

(1) Quantification with the linear calibration curve

Matrix Effect

The assessment of matrix effect was performed by comparing the slope of the curve obtained with three working standard solutions (WSS1 – WSS3 – WSS5), prepared in solvent (HPLC grade water), to the slope obtained with matrix-matched standard solutions (MSS1 – MSS3 – MSS5), prepared in blank matrix (Algal growth medium).
Matrix effect, expressed in % enhancement or suppression of signal, is considered significant if it exceeds ±20%.

The matrix effect was calculated comparing the slope of the curves obtained in matrix and the slope of the curve obtained in solvent.
The result obtained in Algal growth medium expressed in % enhancement of signal is reported below.

Slope of matrix (Algal growth medium)	6671
Slope of solvent (HPLC grade water)	6661
Matrix Effects % (100 * Slope (matrix) / Slope (solvent) – 100)	0.1%

Matrix effects in Algal growth medium, expressed in % enhancement of signal, resulted not significant (lower than 20%), in any case matrix matched standard solutions were used to quantify samples, in order to fully compensate the small difference observed.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection (LOD) is defined as the lowest calibration level standard solution, it was prepared at 2.0 µg/L, as nominal concentration.
The limit of detection (LOD), defined as the lowest calibration solution MSS1, was a final injected solution of Prothioconazole equal to 2.0 µg/L in all matrices.

The limit of quantification (LOQ) is defined as the lowest fortification level solution, it was set at a nominal concentration of 25 µg Test Item / L (corresponding to 7.4 µg Prothioconazole / L).
The limit of quantification (LOQ), defined as the lowest fortification level, was prepared at:
- 7.6 µg/L in algal growth medium (25.6 µg test item/L)*

**taking into account the exact amount weighed for solutions preparation.*

Final extract and Standard Stability
- Extract stability: The stability of the analyte in the final extracts is proven if the recoveries in the fortified samples are within the acceptable range of 70 - 120%, measured against freshly prepared standards.
- Standard stability: The stability of an existing standard (old) is checked by preparing a new standard solution (fresh) and comparing the responses. The means from 5 replicate measurements for each of the two solutions should not differ by more than 10%.

In the following table, the summary of results obtained from the analysis in matrix is reported; they were calculated using the primary transition (quantification transition).

Final Extract Stability	
Matrix	algal growth medium
Time	2 days
Recovery % *(mean value of five replicates)*	82.1
RSD %	9%
Range of recovery	71.4 % – 91.9 %
Standard Stability	
Matrix	algal growth medium
Time	2 days
Mean response old	111035
Mean response fresh	141280
Difference %	- 21.4 % (*)
	Not stable, the standards are daily freshly prepared

(*) differences calculated according to the following formula, which takes in consideration the exact weighed amount of the analytical standard used for preparation of old and fresh standard solutions:

$$\frac{\text{Area "old"} \times \left(\frac{\text{nominal amount to weigh}}{\text{actual weighed amount "old"}}\right) - \text{Area "fresh"} \times \left(\frac{\text{nominal amount to weigh}}{\text{actual weighed amount "fresh"}}\right)}{\text{Area "fresh"} \times \left(\frac{\text{nominal amount to weigh}}{\text{actual weighed amount "fresh"}}\right)}$$

Nominal amount = 10 mg

Actual weighed amount "old" = 9.8 mg
Actual weighed amount "fresh" = 9.8 mg

Conclusion

Due to the obtained results, the analytical method No. 0912-2022 can be considered fully validated according to the SANTE/2020/12830 rev. 1 dated 24/02/21 and it can be considered suitable for the determination of test item residues in the ecotoxicological matrices investigated (algal growth medium).

Matrix: algal growth medium				
Parameter	**Acceptability criteria**	**Result**		
Matrix Effect	Considered significant if ≥ ±20%	0.1 %		
Selectivity / Specificity	untreated blank ≤ 30% LOQ	0 %		
Linearity / Calibration	r ≥ 0.99	*Quantifier transition m/z 344.0 → m/z 326.0* Range 2.0 – 195.8 µg/L, **r = 0.99986**		
		Quantifier transition m/z 344.0 → m/z 154.0 Range 2.0 – 195.8 µg/L, **r = 0.99996**		
		Quantifier transition m/z 344.0 → m/z 189.0 Range 2.0 – 195.8 µg/L, **r = 0.99984**		
		The linearity range 2.0 – 195.8 µg a.i./L corresponds to 6.7 – 659.0 µg test item/L as injected concentration and 6.7 µg test item/L – 131.8 mg test item/L, if injected samples are 200-fold diluted		
LOD	Lowest calibration level	2.0 µg/L		
LOQ	Lowest fortified level	7.6 µg/L		
Stability of final extract	Stability proven if mean recovery between 70% – 120%	82.1 % (2 days) (Low level)		
Stability of standard	Stability proven if fresh and stored standard solutions differ less than ±10%	- 21.4 % (2 days) Not stable, the standards are daily freshly prepared		
Repeatability (Precision) Recovery (Accuracy)		Fortification level	Low (µg/L)	High (mg/L)
		Nominal	7.4	29.71
		Corrected*	7.6	30.30
		Mean found	8.3	31.18
Recovery (Mean between 70% – 120%)			**109.7**	**102.9**
Repeatability (as precision RSD % ≤ 20%)			**9%**	**2%**

(*) corrected for exact test item weighed amount during fortification solution preparation.