REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: FHO04

Product name(s): Prothioconazole/Sulphur (50+625) SC, / Patton Supra

Chemical active substance(s): Prothioconazole 50 g/L, Sulphur 625 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: UPL Holdings Coöperatief U.A.

Submission date: 30/05/2024, updated: October, November 2024

MS Finalisation date: November 2024 (initial Core Assessment)

January 2025 (final Core Assessment)

Version history

When	What
May 2024	Initial dRR – UPL Holdings Coöperatief U.A.
October 2024	Revision 1 updated for the Commission Regulation (EU) 2024/1318 for prothioconazole MRLs.
November 2024	Revision 2 updated for clarification of the studies referred to by the applicant and relied on, but already evaluated at EU peer review.
November 2024	Initial zRMS assessment The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are ~~struck through~~ and shaded for transparency. Following the evaluation and before sending the document for commenting, all coloured highlighting was removed, from the parts updated by the Applicant, for better legibility.
January 2025	Final report (Core Assessment updated following the commenting period) No additional information or assessments after the commenting period.

Table of Contents

5 Analytical methods

5.1 Conclusion and summary of assessment

zRMS conclusions:
Prothioconazole
The endpoints reported in EFSA Scientific Report (2007) 106 are still valid for the ongoing evaluations.
However, taking into account conclusions EFSA regarding residue definitions presented in EFSA Journal 2020;18(2):5999, EFSA Journal 2014;12(5):3689 and EFSA Journal 2018;16(7):5376, based on the metabolic pattern identified in metabolism studies, hydrolysis studies, the toxicological significance of metabolites and degradation products, the residue definitions for plant products were proposed as 'prothioconazole-desthio (sum of isomers)' for enforcement and, as follows, for the risk assessment:
1) sum of prothioconazole-desthio and all metabolites containing the 2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl-2H-1,2,4-triazole moiety, expressed as prothioconazole-desthio (sum of isomers)
2) Triazole alanine (TA) and triazole lactic acid (TLA)
3) Triazole acetic acid (TAA)
4) 1,2,4-triazole (1,2,4-T).
Since all compounds included in the residue definitions are a mixture of enantiomers and since there are no enantiospecific analytical methods, the residue definitions are expressed as "sum of isomers".

Although the residue definition for risk assessment includes consideration of all metabolites containing a common moiety, it is not possible to develop a common moiety method to meet the residue definition for risk assessment. For this reason, all the analytes have to be determined separately. 6 analytes, representing the major portion of the TRR (Total Radioactive Residue) for prothioconazole in the plant metabolism studies, should be determined in residue trials. These are: prothioconazole-desthio, 3-hydroxy-prothioconazole-desthio, 4-hydroxy-prothioconazole-desthio, 5-hydroxy-prothioconazole-desthio, 6-hydroxy-prothioconazoledesthio and alpha-hydroxy-prothioconazole-desthio (including all their acid-hydrolysable conjugates).
The residue definition for enforcement in animal products was set as prothioconazole-desthio (sum of isomers) for all the livestock matrices (EFSA Journal 2014;12(5):3689).
For risk assessment, the residue was defined in all commodities of animal origin as the sum of prothioconazole-desthio and all metabolites containing the 2-(1-chlorocyclopropyl)-3-(2- chlorophenyl)-2-hydroxypropyl-2H-1,2,4-triazole moiety, expressed as prothioconazole-desthio (sum of isomers).

During the peer review under Directive 91/414/EEC, an analytical methods were evaluated and validated for the determination of prothioconazole-desthio in plant matrices and in food of animal origin. The available analytical methods are not enantioselective, hence the sum of isomers will be analyzed (EFSA Journal 2014;12(5):3689).

In EFSA Scientific Report (2007) 106, 1-98, "Conclusion on the peer review of prothioconazole" it is stated that:
„Methods are available to monitor all compounds given in the respective residue definition for food of plant origin, water, soil and air. Residues in food of plant origin can be determined with a multimethod (The German S19 method has been validated for prothioconazole-desthio). Only single methods are available to determine residues of prothioconazole-desthio, in products of animal origin and prothioconazole, prothioconazole-desthio in soil water and air. A method is not available to monitor the glucuronide conjugate in products of animal origin. Also if the active is classified as toxic then methods for body fluids and tissues would need to be considered."

EFSA Scientific Report (2007):
Analytical methods for residues (Annex IIA, point 4.2)

Food/feed of plant origin (principle of method and LOQ for methods for monitoring purposes)	Weeren, Pelz 2000 (GC-MS, JAU6476-desthio) LOQ Wheat, Barley (Forage, Straw): 0.05 mg/kg LOQ Wheat, Barley (Grain), Canola (Seed), Tomato, Orange (Fruit): 0.02 mg/kg
Food/feed of animal origin (principle of method and LOQ for methods for monitoring purposes)	Heinemann 2001b (HPLC-MS/MS, JAU6476-desthio, JAU6476-3 hydroxy-desthio, JAU6476-4-hydroxy-desthio) LOQ Milk: 0.004 mg/kg LOQ Meat, Liver, Kidney, Fat: 0.01 mg/kg Open: there is no method available for the glucuronide conjugate
Soil (principle of method and LOQ)	Schramel 2000 (HPLC-MS/MS, JAU6476, JAU6476-desthio, JAU6476-S-methyl*) * for monitoring not needed LOQ Soil: 0.006 mg/kg Add'l method: Steinhauer 2001 (GC-MS, JAU6476-desthio)

	LOQ Soil: 0.01 mg/kg
Water (principle of method and LOQ)	Sommer 2001b (HPLC-MS/MS, JAU6476, JAU6476-desthio) LOQ Surface and Drinking water: 0.1 µg/L for JAU6476 and 0.05 µg/L for JAU6476-desthio
Air (principle of method and LOQ)	Maasfeld 2002a (HPLC-MS/MS, JAU6476) LOQ Air: 0.015 mg/m^3 Additional method: Maasfeld 2002b (HPLC-MS/MS, JAU6476-desthio) LOQ Air: 0.0006 mg/m^3
Body fluids and tissues (principle of method and LOQ)	Open, data will be required if ECB classify the active as toxic

According to the EFSA Journal 2014;12(5):3689:

Methods for enforcement of residues in food of plant origin

During the peer review under Directive 91/414/EEC, an analytical method using GC-MS and its ILV were evaluated and validated for the determination of prothioconazole-desthio in plant matrices with an LOQ of 0.02 mg/kg in high water content (tomato), high oil content (rape seed), acidic (orange), dry (wheat grain) commodities and an LOQ of 0.05 mg/kg in straw. This method can be confirmed by an independent analytical method using HPLC-MS/MS fully validated for the determination of prothioconazole-desthio in high water content commodities and in straw with an LOQ of 0.05 mg/kg and in high oil content and in dry commodities with an LOQ of 0.01 mg/kg (United Kingdom, 2004). The analytical methods are not enantioselective, hence the sum of isomers will be analyzed.

The multi-residue QuEChERS method in combination with HPLC-MS/MS, as described by CEN (2008), is also available to analyse the prothioconazole-desthio in plant commodities. Nevertheless, the validation data reported are too limited to conclude on the validity of this analytical method (EURL, 2013).
Hence it is concluded that prothioconazole-desthio can be enforced in food of plant origin with an LOQ of 0.02 mg/kg in high oil content and dry commodities and an LOQ of 0.05 mg/kg in high water content commodities and in straw taking into account the highest LOQ of both methods.

Methods for enforcement of residues in food of animal origin

During the peer review under Directive 91/414/EEC, an analytical method using HPLC-MS/MS and its ILV were evaluated and validated for the determination of prothioconazole-desthio only in food of animal origin with an LOQ of 0.004 mg/kg in milk and an LOQ of 0.01 mg/kg in muscle, fat, liver and kidney (United Kingdom, 2004; EFSA, 2007b). Hence it is concluded that prothioconazole-desthio can be enforced in food of animal origin with an LOQ of 0.004 mg/kg in milk and an LOQ of 0.01 mg/kg in muscle, fat, liver and kidney. Nevertheless, prothioconazole-desthio cannot be enforced in eggs. Therefore, ***a fully validated analytical method for the determination of prothioconazole-desthio in eggs is required.***
The available analytical method is not enantioselective, hence the sum of isomers will be analyzed.

The Applicant submitted a number of methods for analysis of residues of prothioconazole for the generation of pre-authorization data and methods for post-authorization control and monitoring purposes.
The details of the evaluation of new and additional studies are referred in Appendix 2.

Since many MRLs have been lowered to 0.01 mg/kg, the validated LOQ of the EU agreed methods by Weeren and Pelz (2000) and Class (2001) are not sufficient to monitor these lowered MRLs for food of plant origin. To cover the current residue definition and MRL limits, the applicant provided the analytical method of Pearson (2022, Report No. QG/20/011) and its ILV (Boubakri, 2023, Report No. S21-08354) for the determination of prothioconazole-desthio residues in/on matrices of plant origin (high water content, high acid, high oil content and high protein/high starch content) with LOQ of 0.005 mg/kg. The ILV is acceptable. The analytical method of Pearson (2022, Report No. QG/20/011) was successfully independently validated. The details of the evaluation of new study is referred in Appendix 2.

According to the conclusions presented in EFSA Journal 2014;12(5):3689, a fully validated analytical method for the determination of prothioconazole-desthio in eggs is required.
The Applicant provided new method (D. Kleinhenz, 2023, Report No. S21-08355) validated for the determination of prothioconazole-desthio (as sum of isomers) in five (milk, egg, fat, liver and meat) different matrices of food of animal origin with the LOQ of 0.004 mg/kg for milk and 0.01 mg/kg for remaining matrices and its ILV (T. Rastogi, 2023, Report No. S21-08868) for the determination of prothioconazole-desthio residues in milk and fat with LOQ of 0.004 mg/kg and 0.01 mg/kg, respectively.
No additional data are required.

According to the EFSA Scientific Report (2007) 106, 1-98, Conclusion on the peer review of Prothioconazole, the point regarding analytical methods for body fluids and tissues for prothioconazole is open, data will be required if ECB classify the active substance as toxic. In Regulation (EU) No 283/2013 it is stated that "*…methods, with a full description, shall be submitted for the analysis in body fluids and tissues for the active substance and relevant metabolites*" and this is a new requirement of SANTE/2020/12830. According to the SANTE/2020/12830: "*Analytical methods for monitoring residues in body fluids and tissues are required for detection of active substances and/or metabolites in humans and animals after possible intoxications or for biomonitoring purposes, regardless of their toxicological classification*."
Therefore, an analytical method for the residues of prothioconazole in body fluids and tissues is required.
The Applicant provided new method (N. Boubakri, 2023, Report No, S21-08361) validated for the determination of prothioconazole-desthio in urine with the LOQ of 0.01 mg/L.
No additional data are required.

Additionally, an independent laboratory validation (ILV) for the method for the determination of residues of prothioconazole in drinking water is missing (in EFSA, 2007). Based on the indication of the SANTE/2020/12830, Rev.2 14. February 2023, the ILV for drinking water should be submitted.
Applicant submitted the LC-MS/MS analytical method (D. Kaiser, 2022, Report No. S21-08359) with its ILV (S. Jooß, 2023, Report No. S21-08869) for the determination of prothioconazole and prothioconazole-desthio in drinking water with LOQ of 0.05 µg/L.
No additional data are required.

Sulphur
Sulphur is a naturally occurring substance. No MRL is set for food of plant or animal origin. Sulphur is included in Annex IV to Regulation (EC) No 396/2005.
Methods for the analysis of residues in food and feed of plant origin, food of animal origin, body fluids and tissues and environmental compartments are not required as no residue definitions were set.

EFSA Scientific Report (2008) 221, 12-70:
Monitoring/Enforcement methods

Food/feed of plant origin (principle of method and LOQ for methods for monitoring purposes)	No MRL, no method required. Nevertheless, one method using HPLC/UV (fully validated) was provided LOQ : 5 mg/kg for cereals LOQ : 10 mg/kg for grapes (not fully validated) Multi residue method (fully validated for grapes with LOQ 0.12 mg/kg and not validated for cereals) However, there was no ILV
Food/feed of animal origin (principle of method and LOQ for methods for monitoring purposes)	No MRL. No method required
Soil (analytical technique and LOQ)	No method required. Nevertheless, one method using HPLC/UV (not validated : no raw data provided and no confirmatory method) was provided
Water (analytical technique and LOQ)	No method required
Air (analytical technique and LOQ)	No method required
Body fluids and tissues (analytical technique and LOQ)	No method required

No additional data for sulphur are required.

Sufficiently sensitive and selective analytical methods are available for the active substance(s) and relevant impurities in the plant protection product.
Noticed data gaps are: none

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

Noticed data gaps are: none.

Commodity/crop	Supported/not supported
Wheat	Supported
Rye	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 for analysis of prothioconazole and sulphur in plant protection product is provided. The following studies have not been evaluated previously to Uniformed Principles and have been provided in support of this assessment.

Comments of zRMS:	Study acceptable. The analytical method for the determination of prothioconazole and sulphur in plant protection product was fully validated according to SANCO/3030/99 rev. 5.

Active substance: Prothioconazole

Reference:	KCP 5.1.1/01
Report	Method(s) validation and determination of Prothioconazole, Sulphur, Prothioconazole-Desthio & Toluene content in one batch of Prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04), V. Buchholz, 2022, Study No. R C1253
Guideline(s):	Yes, Regulation EU 284/2013 and SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole content in the SC formulation was performed by HPLC-PDA using the following method.

HPLC-PDA Conditions

Instrument:	Alliance 2695
Detector:	PDA 2996
Column:	Syncronis C18, 4.6 x 250 mm, 5 µm particle size
Mobile phase A:	Water T1 + 0.1 % phosphoric acid
Mobile phase B:	Acetonitrile + 0.1 % phosphoric acid
Column temperature:	40 ºC
Detection:	PDA at 258 nm
Volume injected:	10 µL
Retention time:	Prothioconazole = ~18.2 min

Linearity
The linearity was evaluated by analysis of the calibration solutions at 7 levels (duplicate injection). The calibration solutions were prepared by weighing approximately 12.5, 17.5, 22.5, 25.5, 27.5, 32.5 and 37.5 mg of the reference item into separate volumetric flasks (50 mL). Approximately 40 mL of acetonitrile was added to each flask. The flasks were placed in an ultrasonic bath for 5 minutes then allowed to cool to room temperature and filled up to the mark with acetonitrile.

Precision
Precision was evaluated by the analysis of 5 calibration solutions at the nominal concentration of the

analyte. The test item solutions were prepared by weighing in quintuplicate; approximately 338 mg of the test item into volumetric flasks (25 mL). Approximately 20 mL of acetonitrile was added to each flask. The flasks were then placed in an ultrasonic bath for 15 minutes and allowed to cool to room temperature and filled up to the mark with acetonitrile.

Accuracy
The accuracy of the method was determined through analysis of blank formulation preparations spiked with the reference item solution at 3 levels.

The blank solution was prepared by weighing in duplicate 338 mg of the blank formulation (FHO04 without prothioconazole) into separate volumetric flasks (25 mL). 20 mL of acetonitrile was the added to each. The flasks were placed into an ultrasonic bath for 15 minutes, allowed to cool to room temperature then filled up to the mark with acetonitrile.

A spiked solution at 80 % of the nominal concentration (~30 g/kg) was prepared by weighing 328 mg of the blank formulation and 10 mg of prothioconazole into separate volumetric flasks (25 mL). 20 mL of acetonitrile was the added to each. The flasks were placed into an ultrasonic bath for 15 minutes, allowed to cool to room temperature then filled up to the mark with acetonitrile.

A spiked solution at 100 % of the nominal concentration (~37 g/kg) was prepared by weighing 325.5 mg of the blank formulation and 12.5 mg of prothioconazole into separate volumetric flasks (25 mL). 20 mL of acetonitrile was the added to each. The flasks were placed into an ultrasonic bath for 15 minutes, allowed to cool to room temperature then filled up to the mark with acetonitrile.

A spiked solution at 120 % of the nominal concentration (~44 g/kg) was prepared by weighing 323 mg of the blank formulation and 15 mg of prothioconazole into separate volumetric flasks (25 mL). 20 mL of acetonitrile was the added to each. The flasks were placed into an ultrasonic bath for 15 minutes, allowed to cool to room temperature then filled up to the mark with acetonitrile.

Validation - Results and discussions
Specificity
The specificity of the method was evaluated by the absence of interference (> 3 %) during the analysis of the blank solution and other reference materials or reagents, by a good separation of the peaks of analytes on the chromatograms of reference items and test item solutions. No interference can be seen in the area of analyte retention time during the analysis of the solvent and blank formulation.

Linearity
A linear response was observed over a concentration range of 18.71 – 55.62 g/kg (25.45 – 75.64 g/L), equivalent to 50.9 – 151.28 % of the nominal prothioconazole concentration within the formulation. An r value of 0.99985 was achieved.

Precision
The precision was assessed by analysis of 5 calibration solutions at the nominal concentration prothioconazole. A mean content of 39.13 g/kg was attained with a %RSD 1.20 % and RSDr of 2.18 %. A Horrat value of 0.55 was attained.

Accuracy
Method accuracy was investigated by spiking a blank formulation with prothioconazole at 80, 100 and 120 % of the nominal concentration of prothioconazole in the formulation. A mean recovery of 100.2, 99.3 and 100.2 % respectively was attained.

Table 5.2-1: Methods suitable for the determination of the active substance prothioconazole in plant protection product FHO04

	Prothioconazole
Author(s), year	V. Buchholz, 2022

	Prothioconazole
Principle of method	Quantitative determination of prothioconazole content in the SC formulation by HPLC-PDA with external standardisation
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	18.71 – 55.62 g/kg (25.45 – 75.64 g/L) 50.9 – 151.28 % Correlation Coefficient: r = 0.99985
Precision – Repeatability Mean n = 5 (%RSD)	Mean content = 39.13 g/kg %RSD = 1.20 % Modified Horwitz: RSDr = 2.18 % Horrat: 0.55 (<1)
Accuracy n = 3 (% Recovery)	80 % = 100.2 % 100 % = 99.3 % 120 % = 100.2 %
Interference/ Specificity	The specificity of the method was evaluated by the absence of interference (> 3 %) during the analysis of the blank solution and other reference materials or reagents, by a good separation of the peaks of analytes on the chromatograms of reference items and test item solutions. No interference can be seen in the area of analyte retention time during the analysis of the solvent and blank formulation.
Comment	None.

Conclusion
The validation criteria for the method used to determine prothioconazole has been met in accordance with SANCO/3030/99 rev. 5.

Active substance: Sulphur

Reference:	KCP 5.1.1/01
Report	Method(s) validation and determination of Prothioconazole, Sulphur, Prothioconazole-Desthio & Toluene content in one batch of Prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04), V. Buchholz, 2022, Study No. R C1253
Guideline(s):	Yes, Regulation EU 284/2013 and SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of sulphur content in the SC formulation was performed by titration using a starch indicator. Sulphur, wet with water and ethanol, was converted to sodium thiosulphate by refluxing with sodium sulphite. The thiosulphate was then titrated with standard iodine solution, in the presence of formaldehyde and acetic acid, using starch as indicator.

Conditions

Ethanol	Reagent grade
Water	Type I (ultra-pure)
Sodium sulphite anhydrous	Reagent grade
Formaldehyde 37%	Reagent grade

Acetic acid	Reagent grade
Starch	Reagent grade
Whatman filter	0.45 µm CME filter

Preparation of starch solution:
Approximately 0.5 g of starch was weighed then added to about 50 mL of water. The solution was boiled for a few minutes, allowed to cool and filtered on Büchner using a Whatman filter. The solution was freshly prepared on each date of titration.

Preparation of acetic acid 20% v/v solution:
20 mL of acetic acid was added into a volumetric flask (100 mL) containing approximately 60 mL of water. It was then filled up to the mark with water and let to stand for 5 minutes.

Preparation of solutions for thiosulphate correction:
Thiosulphate initially present in sulphur must be determined and subtracted from the total sulphur amount found. Weigh (to the nearest 0.01 mg) approximately 530 mg of the test item into a 250 mL volumetric flask, fill up to the mark with water and mix thoroughly. Centrifuge the suspension for 30 min at 3000 rpm then filter through a 0.45 µm CME filter. Then titrate with Iodine solution.

Linearity
The linearity was evaluated by the titration of reference solutions at 7 levels. The reference solutions were prepared by weighing approximately 129, 177, 224, 249, 275, 325 and 379 mg of the reference item of Sulphur and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was attached, the mixture warmed slowly to dissolve the sulphur, then boiled for 1.5 hr whilst stirring with a magnetic stirrer. After all the sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

Precision
The precision of the method was assessed through the fivefold titration of the active ingredient in the test item (five independent weights). The test item solutions were prepared by weighing in quintuplicate approximately 530 mg of the test item and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was added, the mixture warmed slowly to dissolve the sulphur, then boiled for 1 hr whilst stirring with a magnetic stirrer. After all the sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

Accuracy
The accuracy of the method was determined through the titration of blank formulation preparations spiked with reference item solution at 3 levels.

The blank solution was prepared by weighing in duplicate approximately 530 mg of the blank formulation (FHO04 without sulphur) and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was added, the mixture warmed slowly to dissolve the sulphur, then boiled for 1 hr whilst stirring with a magnetic stirrer. After all the sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

A spiked solution at 80 % of the nominal concentration (~368 g/kg) was prepared by weighing approximately 343 mg of the blank formulation and 200 mg of the reference item of Sulphur and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was added, the mixture warmed slowly to dissolve the sulphur, then boiled for 1 hr whilst stirring with a magnetic stirrer. After all the

sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

A spiked solution at 100 % of the nominal concentration (~460 g/kg) was prepared by weighing approximately 293 mg of the blank formulation and 250 mg of the reference item of Sulphur and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was added, the mixture warmed slowly to dissolve the sulphur, then boiled for 1 hr whilst stirring with a magnetic stirrer. After all the sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

A spiked solution at 120 % of the nominal concentration (~552 g/kg) was prepared by weighing approximately 243 mg of the blank formulation and 300 mg of the reference item of Sulphur and transferred to a 250 mL round bottom flask. The sample was wetted thoroughly with 25 mL of ethanol. 35 mL of water and 2.5 g of sodium sulphite anhydrous were added. A reflux condenser was added, the mixture warmed slowly to dissolve the sulphur, then boiled for 1 hr whilst stirring with a magnetic stirrer. After all the sulphur was dissolved, the solution was cooled to room temperature. The solution was then transferred quantitatively to a volumetric flask (250 mL), filled up to the mark with water and mixed thoroughly.

Validation - Results and discussions

Specificity

The specificity of the method was determined by the titration of reagents and the blank formulation. When titrating the reagents and the blank formulation, a low volume of titration can be seen. This volume is a negligible interference (< 3% of the nominal volume).

Linearity

A linear response was observed over a concentration range of 236 – 730 g/kg (321.0 – 992.8 g/L), equivalent to 51.4 – 158.9 % of the nominal sulphur concentration within the formulation. An r value of 0.99814 was achieved.

Precision

The precision was assessed by analysis of 5 calibration solutions at the nominal concentration sulphur. A mean content of 469.8 g/kg was attained with a %RSD 1.10 % and RSDr of 1.50 %. A Horrat value of 0.73 was attained.

Accuracy

Method accuracy was investigated by spiking a blank formulation with sulphur at 80, 100 and 120 % of the nominal concentration of prothioconazole in the formulation. A mean recovery of 101.5, 102.7 and 102.2 % respectively was attained.

Table 5.2-2: Methods suitable for the determination of the active substance sulphur in plant protection product FHO04

	Sulphur
Author(s), year	V. Buchholz, 2022
Principle of method	Quantitative determination of sulphur content in the SC formulation by titration (in principle, CIPAC method 18, handbook E, pages 202-204)
Linearity **(linear between** **mg/L / % range of the declared content)** **(correlation coefficient, expressed as r)**	236 – 730 g/kg (321.0 – 992.8 g/L) 51.4 – 158.9 % Correlation Coefficient: r = 0.99814
Precision – Repeatability Mean **n = 5** **(%RSD)**	Mean content = 469.8 g/kg %RSD = 1.10 % Modified Horwitz: RSDr = 1.50 % Horrat: 0.73 (<1)

	Sulphur
Accuracy **n = 3** **(% Recovery)**	80 % = 101.5 % 100 % = 102.7 % 120 % = 102.2 %
Interference/ Specificity	The specificity of the method was determined by the titration of reagents and the blank formulation. When titrating the reagents and the blank formulation, a low volume of titration can be seen. This volume is a negligible interference (< 3% of the nominal volume).
Comment	None.

Conclusion
The validation criteria for the method used to determine sulphur has been met in accordance with SANCO/3030/99 rev. 5.

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

An overview on the acceptable methods and possible data gaps for analysis of relevant impurities in plant protection product is provided as follows. The following studies have not been evaluated previously to Uniformed Principles and have been provided in support of this assessment.

Comments of zRMS:	Study acceptable The analytical method for the determination of relevant impurities in plant protection product was fully validated according to SANCO/3030/99 rev. 5.

Relevant impurity: Toluene and Prothioconazole-Desthio

Reference:	KCP 5.1.1/01
Report	Method(s) validation and determination of Prothioconazole, Sulphur, Prothioconazole-Desthio & Toluene content in one batch of Prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04), V. Buchholz, 2022, Study No. R C1253
Guideline(s):	Yes, Regulation EU 284/2013 and SANCO/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of toluene and prothioconazole-desthio content in the SC formulation was performed by HPLC-PDA using the following method.

<u>HPLC-PDA Conditions</u>

Instrument:	Alliance 2695
Detector:	PDA 2998
Column:	Xbridge, 4.6 x 150 mm, 5 µm particle size
Mobile phase A:	Water T1 + 0.1 % phosphoric acid
Mobile phase B:	Methanol 100%
Column temperature:	40 ºC

Detection:	PDA at 210 nm
Volume injected:	20 µL
Retention time:	Toluene = ~7.7 min
	Prothioconazole-desthio = ~11.6 min

Linearity

The linearity was evaluation by the analysis of the calibration solutions at 9 levels (single injection). A stock solution was prepared by weighing approximately 20 mg of each reference item into separate volumetric flasks (20 mL). Approximately 15 mL of methanol was added to each flask then the flasks were placed in an ultrasonic bath for 5 minutes, allowed to cool to room temperature, and filled up to the mark with methanol.

An aliquot of the prothioconazole-desthio stock solution was diluted in methanol to obtain an intermediate solution at a concentration of approximately 100 µg/mL.
Aliquots of the stock solution of Toluene and of the intermediate solution of Prothioconazole-desthio were diluted in methanol to obtain solutions at suitable concentrations ranging from 0.25 to 2.5 µg/mL for prothioconazole-desthio and ranging from 2.5 to 25 µg/mL for toluene.

Precision

Precision was evaluated by the analysis of 5 calibration solutions. The test item solutions containing 10 mg/kg prothioconazole-desthio and 0.10 g/kg toluene were prepared by weighing in quintuplicate approximately 1000 mg of the blank formulation and adding 0.10 mL of each spiking solution into separate volumetric flasks (10 mL). Approximately 8 mL of methanol was added to each flask. The flasks were then placed in an ultrasonic bath for 15 minutes and allowed to cool to room temperature and filled up to the mark with methanol. The solutions were filtered with a PTFE 0.45 µm before analysis.

Accuracy

The accuracy of the method was determined through the determination of blank formulation preparations spiked with reference item solution at 2 levels.

The blank solution was prepared by weighing 1000 mg of the blank formulation (FHO04 without prothioconazole) into separate volumetric flasks (10 mL). Approximately 8 mL of methanol was added to each flask. The flasks were then placed in an ultrasonic bath for 15 minutes and allowed to cool to room temperature and filled up to the mark with methanol. The solutions were filtered with a PTFE 0.45 µm before analysis.

Spiked solutions containing 15 mg/kg prothioconazole-desthio and 0.15 g/kg toluene were prepared by weighing in quintuplicate approximately 1000 mg of the blank formulation and adding 0.15 mL of each spiking solution into separate volumetric flasks (10 mL). Approximately 8 mL of methanol was added to each flask. The flasks were then placed in an ultrasonic bath for 15 minutes and allowed to cool to room temperature and filled up to the mark with methanol. The solutions were filtered with a PTFE 0.45 µm before analysis.

Spiked solutions containing 20 mg/kg prothioconazole-desthio and 0.2 g/kg toluene were prepared by weighing in quintuplicate approximately 1000 mg of the blank formulation and adding 0.2 mL of each spiking solution into separate volumetric flasks (10 mL). Approximately 8 mL of methanol was added to each flask. The flasks were then placed in an ultrasonic bath for 15 minutes and allowed to cool to room temperature and filled up to the mark with methanol. The solutions were filtered with a PTFE 0.45 µm before analysis.

Validation - Results and discussions

Specificity

The specificity of the method was evaluated by the absence of interference during the analysis of the blank solution and other reference materials or reagents, by a good separation of the peaks of analytes on the chromatograms of reference items and test item solutions.

Linearity
A linear response was observed for toluene over a concentration range of 0 – 0.25 g/kg (0 – 0.34 g/L), equivalent to 0 – 130.8 % of the nominal toluene concentration within the formulation. An r value of 0.99996 was achieved. A linear response was also observed for prothioconazole-desthio over a concentration range of 0 – 25 mg/kg (0 – 0.34 g/L), equivalent to 0 – 1133.3 % of the nominal prothioconazole-desthio concentration within the formulation. An r value of 0.99893 was achieved.

Precision
The precision was assessed by analysis of 5 calibration solutions containing 10 mg/kg of prothioconazole-desthio and 0.10 g/kg of toluene. For toluene, a mean content of 0.095 g/kg was attained with a %RSD 3.21 % and RSDr of 5.40 %. A Horrat value of 0.59 was attained. For prothioconazole-desthio, a mean content of 9.48 mg/kg was attained with a %RSD 2.04 % and RSDr of 7.64 %. A Horrat value of 0.27 was attained.

Accuracy
Method accuracy was investigated by spiking a blank formulation with 0.15 g/kg and 0.20 g/kg of toluene. A mean recovery of 101.9 and 102.4 % respectively was attained. Another blank formulation was spiked with 15 mg/kg and 20 mg/kg of prothioconazole-desthio. A mean recovery of 103.5 and 101.8 % respectively was attained

Table 5.2-3: Methods suitable for the determination of the relevant impurities toluene and prothioconazole-desthio in plant protection product (PPP) FHO04

	Toluene	**Prothioconazole-desthio**
Author(s), year	V. Buchholz, 2022	
Principle of method	Quantitative determination of toluene and prothioconazole-desthio content in the SC formulation was performed by HPLC-PDA	
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	0 – 0.25 g/kg (0 – 0.34 g/L) 0 – 130.8 % r = 0.99996	0 – 25 mg/kg (0 – 0.34 g/L) 0 – 1133.3 % r = 0.99893
Precision – Repeatability Mean n = 5 (%RSD)	Mean content = 0.095 g/kg %RSD = 3.21 % Modified Horwitz: RSDr = 5.40 % Horrat: 0.59 (<1)	Mean content = 9.48 mg/kg %RSD = 2.04 % Modified Horwitz: RSDr = 7.64 % Horrat: 0.27 (<1)
Accuracy n = 3 (% Recovery)	~0.15 g/kg = 101.9 % (RSD = 0.57 %) ~0.20 g/kg = 102.4 % (RSD = 0.82 %)	~15 mg/kg = 103.5 % (RSD = 5.33 %) ~20 mg/kg = 101.8 % (RSD = 5.08 %)
Interference/ Specificity	The specificity of the method was evaluated by the absence of interference during the analysis of the blank solution and other reference materials or reagents, by a good separation of the peaks of analytes on the chromatograms of reference items and test item solutions.	
LOQ	LOD = 0.002 g/kg LOQ = 0.1 g/kg	LOD = 2.5 mg/kg LOQ = 10 mg/kg
Comment	None.	

Conclusion
The validation criteria for the method used to determine the relevant impurities toluene and prothioconazole-desthio has been met in accordance with SANCO/3030/99 rev. 5.

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

Not required. None of the formulants are of toxicological concern. Please refer to Part C for additional information.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

No applicable CIPAC methods available for the determination of prothioconazole in SC formulations. Please refer to Point 5.2.1.1 where a CIPAC method was used for the determination of sulphur in FHO04.

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 and sulphur for the generation of pre-authorization data is given in the following table. For the detailed evaluation of new/additional studies it is referred to Appendix 2.

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

Component of residue definition: prothioconazole*				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Plants (Residues) Wheat (whole plant, grain, straw)	Primary and confirmatory*	0.005 mg/kg (prothioconazole-desthio) 0.005 mg/kg (hydroxy metabolites of prothioconazole-desthio) 0.005-0.05 mg/kg (TDMs)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011, KCP 5.1.2/01 D. Giancola, 2023, Report No. QG/22/001, KCP 5.1.2/02 B. Phipps, 2024, Report No. QG/20/012, KCP 5.1.2/03
Animal products, food of animal origin (Residues)	- Note: Honey is not relevant for the intended uses of the product			
Soil, water, sediment (Environmental fate)	-			
Soil, water (Efficacy)	-			
Feed, body fluids (Toxicology)	-			
Body fluids, air (Exposure)	-			
Reconstituted water (Ecotoxicology)	Primary and confirmatory	4.052 µg/L	LC-MS/MS	M. Patel, 2023, Report No. 228-2-13-29109, KCP 5.1.2/04
Algal media (Ecotoxicology)	Primary and confirmatory	4.052 µg/L	LC-MS/MS	M. Patel, 2023, Report No. 228-2-13-29109, KCP 5.1.2/04
Stock solutions 50 % (w/v) aqueous sucrose (Ecotoxicology)	Primary and confirmatory	15.7 mg/L	HPLC-MS/MS	D., Ripperger, 2022, Report No. S21-06042, KCP 5.1.2/05 (filed in KCP 10.3.1.1/02)
Stock solutions 0.1 % Triton X 100 (Ecotoxicology)	Primary and confirmatory	78.3 mg/L	HPLC-MS/MS	D., Ripperger, 2022, Report No. S21-06042, KCP 5.1.2/05 (filed in KCP 10.3.1.1/02)
Feeding studies 50 % (w/v) aqueous sucrose solution + 0.1 % xanthan gum (Ecotoxicology)	Primary and confirmatory	7.56 mg/kg	LC-MS/MS	T. Ansaloni, 2022, Report No. S21-06044, KCP 5.1.2/06 (filed in KCP 10.3.1.2/01)
Feeding studies larval diet (Ecotoxicology)	Primary and confirmatory	0.0783 mg/kg	LC-MS/MS	T. Ansaloni, 2022, Report No. S21-06046, KCP 5.1.2/07 (filed in KCP 10.3.1.3/01)
Spray solutions, petri dishes (Ecotoxicology)	Primary and confirmatory	0.01 mg/kg	LC-MS/MS	T. Vollmer, 2023, Report No. S21-03781, KCP 5.1.2/08 (filed in KCP 10.4.1.2/01)

Component of residue definition: prothioconazole*				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Soil samples (Ecotoxicology)	Primary and confirmatory**	0.01 mg/kg	LC-MS/MS	T. Vollmer, 2023, Report No. S21-03781, KCP 5.1.2/08 (filed in KCP 10.4/02)
Tap water (Ecotoxicology)	Primary and confirmatory	392 mg/L	HPLC-MS/MS	D. Ripperger, 2022, Report No. S21-05533, KCP 5.1.2/09 (filed in KCP 10.6.2/01) D. Ripperger, 2023, Report No. S21-05534, KCP 5.1.2/10 (filed in KCP 10.6.1/01)
Standard waters (Properties)	Primary and confirmatory	0.005 mg/mL	HPLC-PDA	V. Buchholz, 2022, Report No. R C1254, KCP 5.1.2/11 (filed in KCP 2.1)

*The components of the residue definition are:

1) Sum of prothioconazole-desthio and all metabolites containing the 2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl-2H-1,2,4-triazole moiety, expressed as prothioconazole-desthio (sum of isomers);
2) Triazole alanine (TA) and triazole lactic acid (TLA);
3) Triazole acetic acid (TAA);
4) 1,2,4-triazole (1,2,4-T).

** The components of the residue definition are prothioconazole and prothioconazole-desthio and s-methyl prothioconazole

Component of residue definition: sulphur				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Plants, plant products,... (Residues)	n/a	n/a	No MRL; no method required	Reg. (EC) No 459/2010
Animal products, food of animal origin,... (Residues)	n/a	n/a	No MRL; no method required	Reg. (EC) No 459/2010
Soil, water, sediment,... (Environmental fate)	n/a	n/a	The active substance is naturally occurring and it would be impossible to distinguish between natural occurrence and the amount resulting from pesticide usage	EFSA Scientific Report (2008) 221, 1-70
Soil, water,... (Efficacy)	n/a	n/a		
Feed, body fluids,... (Toxicology)	n/a	n/a	No MRL; no method required	Reg. (EC) No 459/2010
Body fluids, air,.... (Exposure)	n/a	n/a	No MRL; no method required	Reg. (EC) No 459/2010
Reconstituted water (Ecotoxicology)	Primary and confirmatory	12.732 mg/L	LC-MS/MS	M. Patel, 2023, Report No. 228-2-13-29109, KCP 5.1.2/04
Algal media (Ecotoxicology)	Primary and confirmatory	10.115 mg/L	LC-MS/MS	M. Patel, 2023, Report No. 228-2-13-29109, KCP 5.1.2/04
Stock solutions 50 % (w/v) aqueous sucrose (Ecotoxicology)	Primary and confirmatory	475 mg/L	HPLC-MS/MS	D., Ripperger, 2022, Report No. S21-06042, KCP 5.1.2/05 (filed in KCP 10.3.1.1/02)

Component of residue definition: sulphur				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Stock solutions 0.1 % Triton X 100 (Ecotoxicology)	Primary and confirmatory	1412 mg/L	HPLC-MS/MS	D., Ripperger, 2022, Report No. S21-06042, KCP 5.1.2/05 (filed in KCP 10.3.1.1/02)
Feeding studies 50 % (w/v) aqueous sucrose solution + 0.1 % xanthan gum (Ecotoxicology)	Primary and confirmatory	27.4 mg/kg	LC-MS/MS	T. Ansaloni, 2022, Report No. S21-06044, KCP 5.1.2/06 (filed in KCP 10.3.1.2/01)
Tap water (Ecotoxicxology)	Primary and confirmatory	4710 mg/L	HPLC-MS/MS	D. Ripperger, 2022, Report No. S21-05533, KCP 5.1.2/09 (filed in KCP 10.6.2/01) D. Ripperger, 2023, Report No. S21-05534, KCP 5.1.2/10 (filed in KCP 10.6.1/01)
Standard waters (Properties)	Primary and confirmatory	0.005 mg/mL	HPLC-PDA	V. Buchholz, 2022, Report No. R C1254, KCP 5.1.2/11 (filed in KCP 2.1)

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substance and relevant impurities are the same as those utilised within pre-authorisation.

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

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

The current legal residue definition per Regulation (EU) 2024/1318:
Prothioconazole: prothioconazole-desthio (sum of isomers)

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 – ~~wheat (forage)~~	Prothioconazole-desthio (sum of isomers)	0.01* mg/kg	Reg. (EU) 2024/1318
~~Plant, high water content – rye (forage)~~		~~0.05 mg/kg~~	~~Reg. (EU) 2024/1318~~
Plant, high acid content		~~Not relevant for the intended uses of this product~~ 0.01 mg/kg*	Reg. (EU) 2024/1318
Plant, high protein/high starch content (dry commodities) – ~~wheat (grain)~~		0.01* mg/kg	Reg. (EU) 2024/1318
~~Plant, high protein/high starch content (dry commodities) – wheat~~		~~0.1 mg/kg~~	~~Reg. (EU) 2024/1318~~

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
~~(straw)~~			
~~Plant, high protein/high starch content (dry commodities) – rye (grain)~~		~~0.05 mg/kg~~	~~Reg. (EU) 2024/1318~~
~~Plant, high protein/high starch content (dry commodities) – rye (straw)~~		~~0.1 mg/kg~~	~~Reg. (EU) 2024/1318~~
Plant, high oil content		~~Not relevant for the intended uses of this product~~ 0.02 mg/kg*	Reg. (EU) 2024/1318
Plant, difficult matrices (hops, spices, tea)		~~Not relevant for the intended uses of this product~~ 0.05 mg/kg*	Reg. (EU) 2024/1318
Muscle	Prothioconazole-desthio (sum of isomers)	0.01 mg/kg	Reg. (EU) 2024/1318
Milk		0.01* mg/kg	Reg. (EU) 2024/1318
Eggs		0.01* mg/kg	Reg. (EU) 2024/1318
Fat		0.01* ~~–0.02~~ mg/kg	Reg. (EU) 2024/1318
Liver, kidney		0.1~~– 0.5~~ mg/kg	Reg. (EU) 2024/1318
Honey		0.05* mg/kg	Reg. (EU) 2024/1318
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	RAC 13 µg/L (prothioconazole) RAC 0.334 µg/L (prothioconazole-desthio)	from EC50 for *Daphnia magna*, F. Heimbach 1999, Report No. HBF/DM 212, DAR, prothioconazole, 2004, IIA 8.2.4/01 (ESFA Scientific Report, 2007) from NOEC for *Oncorhynchus mykiss* (ELS), T. Gries, 2002, Report No. 102.013.321, DAR, prothioconazole, 2004, IIA 8.2.2.2/02 (ESFA Scientific Report, 2007)
Air	Prothioconazole and prothioconazole-desthio	LOQ = 0.15 µg/m^3 (prothioconazole) LOQ = 0.0006 µg/m^3 (prothioconazole- desthio)	W. Maasfeld 2002a and 2002b, Report Nos. 00724 and 00731, DAR Addendum, prothioconazole, 2007, IIA 4.2.4.1/01 and IIA 4.2.4.1/02 (EFSA Scientific Report, 2007)
Tissue (meat or liver)	Not allocated	Not required	Open point in EFSA Scientific Report (2007). A new method for prothioconazole-desthio with LOQ = 0.01 mg/kg is presented in Section 5.3.2.7
		0.01 mg/kg	SANTE/2020/12830, Rev.2
Body fluids		Not required	Open point in EFSA Scientific Report (2007). A new method for prothioconazole-desthio with LOQ = 0.01 mg/kg is presented in Section 5.3.2.7

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
		0.01 mg/L	SANTE/2020/12830, Rev.2

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. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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

Component of residue definition: prothioconazole-desthio				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary	0.05 mg/kg (wheat, forage) 0.05 mg/kg (barley, forage) 0.02 mg/kg (tomato)	GC-MS	R.D. Weeren and S. Pelz, 2000, Report No. 00086/M033, DAR prothioconazole, 2004, IIA 4.2.1.1/06 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (cucumber, wheat forage, barley forage)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)
	ILV	0.02 mg/kg (tomato fruit)	GC-MS	Th. Class, 2001, Report No. P/B 484 G, DAR prothioconazole, 2004, IIA 4.2.1.1/07 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (cucumber)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354, KCP 5.2/02
	Confirmatory	Not required		
High acid content	Primary	0.02 mg/kg (orange)	GC-MS	R.D. Weeren and S. Pelz, 2000, Report No. 00086/M033, DAR prothioconazole, 2004, IIA 4.2.1.1/06 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (orange)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)
	ILV	0.02 mg/kg (tomato fruit)	GC-MS	Th. Class, 2001, Report No. P/B 484 G, DAR prothioconazole, 2004, IIA 4.2.1.1/07 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (orange)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354, KCP 5.2/02
	Confirmatory	Not required		
High oil content	Primary	0.02 mg/kg (rapeseed)	GC-MS	R.D. Weeren and S. Pelz, 2000, Report No. 00086/M033, DAR

				prothioconazole, 2004, IIA 4.2.1.1/06 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)
	ILV	0.02 mg/kg (rape seed)	GC-MS	Th. Class, 2001, Report No. P/B 484 G, DAR prothioconazole, 2004, IIA 4.2.1.1/07 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (oilseed rape seed)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354, KCP 5.2/02
	Confirmatory	Not required		
High protein/high starch content (dry)	Primary	0.05 mg/kg (wheat, straw) 0.02 mg/kg (wheat, grain) 0.05 mg/kg (barley, straw) 0.02 mg/kg (barley, grain)	GC-MS	R.D. Weeren and S. Pelz, 2000, Report No. 00086/M033, DAR prothioconazole, 2004, IIA 4.2.1.1/06 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.005 mg/kg (dried field bean, wheat straw, barley straw)	LC-MS/MS	J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)
	ILV	0.02 mg/kg (cereal grain)	GC-MS	Th. Class, 2001, Report No. P/B 484 G, DAR prothioconazole, 2004, IIA 4.2.1.1/07 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.005 mg/kg (dried field bean)	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08354, KCP 5.2/02
	Confirmatory	Not required		
Difficult (if required, depends on intended use)	Primary	Not relevant for the intended uses of this product		

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

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	DAR – Prothioconazole 2004. Extraction efficiency was demonstrated.

Extraction efficiency of the residue method in cereals and rape (Heinemann, O., 2001a, Report No. 00647, IIA 4.2.1.1/03) was evaluated using aged radioactive residues from the metabolism study on wheat (Haas, M., 2001, Report No. MR-084/01, IIA 6.1.1.1/01). In summary, the comparison of the residue analytical method of extraction for plant matrices with the extraction method used in the metabolism study demonstrated the suitability of the analytical method (extracting with an acetonitrile/water solvent system) for the determination of the relevant residue in plant matrices. No further consideration was determined to be necessary and the extraction efficacy will be reevaluated at renewal.

zRMS comments:
The EU agreed methods by Weeren and Pelz (2000) and Class (2001) are not sufficient to monitor lowered MRLs than 0.02 mg/kg for food of plant origin. Please refer to the zRMS-PL conclusions presented in point 5.1.

The applicant provided sufficiently validated analytical method of J. Pearson (2022, Report No. QG/20/011) and the ILV of QG/20/011 (N. Boubakri, 2023, Report No. S21-08354) for the determination of relevant residues of prothioconzole-desthio in/on matrices of plant origin with LOQ of 0.005 mg/kg. The methods are acceptable. The details of the evaluation of new and additional studies are referred in Appendix 2.

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. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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

Component of residue definition: prothioconazole-desthio*				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk	Primary	0.004 mg/kg	HPLC-MS/MS	O. Heinmann, 2001b, Report No. 00655/M001, DAR prothioconazole, 2004, IIA 4.2.1.1/05 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.004 mg/kg	HPLC-MS/MS	L. Dubey, 2001, Report No. A-14-01-01, DAR prothioconazole, 2004, IIA 4.2.1.1/08 EU agreed (EFSA Scientific Report, 2007)
Eggs	Primary	0.01 mg/kg	LC-MS/MS	D. Kleinhenz, 2023, Report No. S21-08355, KCP 5.2/03 New data
	ILV	0.01 mg/kg	LC-MS/MS	T. Rastogi, 2023, Report No. S21-08868, KCP 5.2/04 New data
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	O. Heinmann, 2001c, Report No. 00655, DAR prothioconazole, 2004, IIA 4.2.1.1/04 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	L. Dubey, 2001, Report No. A-14-01-01, DAR prothioconazole, 2004, IIA 4.2.1.1/08 EU agreed (EFSA Scientific Report, 2007)
Fat	Primary	0.01 mg/kg	HPLC-MS/MS	O. Heinmann, 2001c, Report No. 00655, DAR prothioconazole, 2004, IIA 4.2.1.1/04 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	L. Dubey, 2001, Report No. A-14-01-

Component of residue definition: prothioconazole-desthio*				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
				01, DAR prothioconazole, 2004, IIA 4.2.1.1/08 EU agreed (EFSA Scientific Report, 2007)
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	O. Heinmann, 2001c, Report No. 00655, DAR prothioconazole, 2004, IIA 4.2.1.1/04 EU agreed (EFSA Scientific Report, 2007)
	ILV	0.01 mg/kg	HPLC-MS/MS	L. Dubey, 2001, Report No. A-14-01-01, DAR prothioconazole, 2004, IIA 4.2.1.1/08 EU agreed (EFSA Scientific Report, 2007)
Honey	Primary	0.005 mg/kg	LC-MS/MS	J. Hitchens, 2023, Report No. QG/21/009, KCP 5.2/05 New data
	ILV	0.005 mg/kg	LC-MS/MS	J. Wagner, 2023, Report No. S21-08357, KCP 5.2/06 New data

*An alternative residue definition for animal matrices was proposed the EFSA Scientific Report 2007: Sum of prothioconazole-desthio and its glucuronic conjugate, expressed as prothioconazole-desthio. However, in the EFSA Art 12 MRL review 2014, the residue definition was instead proposed as prothioconazole-desthio.

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

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	DAR – Prothioconazole 2004. Extraction efficiency was demonstrated.

Extraction efficiency of the residue method in cereals and rape (Heinemann, O., 2001a, Report No. 00647, IIA 4.2.1.1/03) was evaluated using aged radioactive residues from the metabolism study on wheat (Haas, M., 2001, Report No. MR-084/01, IIA 6.1.1.1/01). In summary, the comparison of the residue analytical method of extraction for plant matrices with the extraction method used in the metabolism study demonstrated the suitability of the analytical method (extracting with an acetonitrile/water solvent system) for the determination of the relevant residue in plant matrices. No further consideration was determined to be necessary and the extraction efficacy will be reevaluated at renewal.

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 tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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

Component of residue definition: prothioconazole and prothioconazole-desthio			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.006 mg/kg	HPLC-MS/MS	O. Schramel, 2000, Report No. 00610, DAR prothioconazole, 2004, IIA 4.2.2.1/01 EU agreed (EFSA Scientific Report, 2007)
Primary	0.01 mg/kg (prothioconazole-desthio only)	GC-MS	S. Steinhaumer, 2001, Report No. 00086/M038, DAR prothioconazole, 2004, IIA 4.2.2.1/03 EU agreed (EFSA Scientific Report, 2007)
Primary	0.006 mg/kg (prothioconazole) 0.002 mg/kg (prothioconazole-desthio)	LC-MS/MS	M. Kaiser, 2022, Report No. S21-08358, KCP 5.2/07 New data

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

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. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	H. Sommer, 2001, Report No. 00684, DAR, prothioconazole, 2004, IIA 4.2.3.1/03 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.05 µg/L	LC-MS/MS	D. Kaiser, 2022, Report No. S21-08359, KCP 5.2/08 New data
	ILV	0.05 µg/L	LC-MS/MS	S. Jooß, 2023, Report No. S21-08869 New data
Surface water	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	H. Sommer, 2001, Report No. 00684, DAR, prothioconazole, 2004, IIA 4.2.3.1/03 EU agreed (EFSA Scientific Report, 2007)
	Primary	0.05 µg/L	LC-MS/MS	D. Kaiser, 2022, Report No. S21-08359, KCP 5.2/08 New data

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

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. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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	0.015 µg/L (prothioconazole) 0.0006 µg/L (prothioconazole-desthio)	HPLC-MS/MS	W. Maasfeld 2002a and 2002b, Report Nos. 00724 and 00731, DAR Addendum, prothioconazole, 2007, IIA 4.2.4.1/01 and IIA 4.2.4.1/02 EU agreed, EFSA Scientific Report, 2007

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

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

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

Table 5.3-9: Methods for body fluids and tissues (if appropriate)

Component of residue definition: prothioconazole-desthio			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.01 mg/L	LC-MS/MS	N. Boubakri, 2023, Report No. S21-08361, KCP 5.2/10 New data

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

5.3.2.8 Other studies/ information

None submitted.

5.3.3 Description of analytical methods for the determination of residues of sulphur (KCP 5.2)

Sulphur is a naturally occurring substance. According to the DAR, it would be impossible to distinguish between natural occurrence and the amount resulting from pesticide usage. Thus, post-authorization and monitoring methods are not relevant and not required. This is confirmed by the EFSA Conclusion (2008), where residue definitions for soil, water and air were considered not relevant and monitoring/enforcement methods for soil, water and air were not required. Furthermore, according to EFSA (2008), no MRLs were

required for plant and animal products, and monitoring/enforcement methods products of plant and animal origin were also not required. Sulphur is included in Annex IV to Regulation (EC) No 396/2005.

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

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

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

Not relevant, no MRL is set for food of plant or animal origin. Sulphur is included in Annex IV to Regulation (EC) No 396/2005.

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

Not relevant, no MRL is set for food of plant or animal origin. Sulphur is included in Annex IV to Regulation (EC) No 396/2005.

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

Not relevant. The active substance is composed of natural occurring compounds. It would be impossible to distinguish between what occurs naturally and what occurs as a result of pesticide usage. Methods for naturally occurring non-toxic substances are usually not required (SANCO/825/00 rev.8.1). This is confirmed by the EFSA Conclusion (2008), where a residue definition for soil was considered not relevant and a monitoring/enforcement method for soil was not required.

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

Not relevant. The active substance is composed of natural occurring compounds. It would be impossible to distinguish between what occurs naturally and what occurs as a result of pesticide usage. Methods for naturally occurring non-toxic substances are usually not required (SANCO/825/00 rev.8.1). This is confirmed by the EFSA Conclusion (2008), where a residue definition for water was considered not relevant and a monitoring/enforcement method for water was not required.

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

Not relevant. The active substance is composed of natural occurring compounds. It would be impossible to distinguish between what occurs naturally and what occurs as a result of pesticide usage. Methods for naturally occurring non-toxic substances are usually not required (SANCO/825/00 rev.8.1). This is confirmed by the EFSA Conclusion (2008), where a residue definition for air was considered not relevant and a monitoring/enforcement method for air was not required.

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

Not relevant, the active substance is not classified as toxic or very toxic. This is confirmed by the EFSA Conclusion (2008), where a monitoring/enforcement method for body fluids and tissues was not required.

5.3.3.8 Other studies/ information

Not required.

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	Vertebrate study Y/N	Owner
KCP 5.1.1/01	V. Buchholz	2022	Method(s) validation and determination of Prothioconazole, Sulphur, Prothioconazole-Desthio & Toluene content in one batch of Prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04 Company Report No R. C1253 GLP Unpublished	N	UPL
KCP 5.1.2/01	J. Pearson	2022	Prothioconazole-desthio: Method Validation in Crops Company Report No. QG/20/011 GLP Unpublished	N	UPL
KCP 5.1.2/02	D. Giancola	2023	Hydroxy Metabolites of prothioconazole-desthio: Method Validation in Crops Report No. QG/22/001 GLP Unpublished	N	UPL
KCP 5.1.2/03	B. Phipps	2024	Triazole Derived Metabolites (TDM's) (1,2,4-Ttriazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops Report No. QG/20/012 GLP Unpublished	N	UPL
KCP 5.1.2/04	M. Patel	2023	Validation of the Analytical Method for Determination of Active Substance Concentration and Stability of Prothioconazole + Sulphur in Matrix, Following the Application of Prothioconazole/Sulphur (50 + 625) G/L SC Report No. 228-2-13-29109 GLP Unpublished	N	UPL
KCP 5.1.2/05 (filed in KCP 10.3.1.1/02)	D. Ripperger	2022	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Acute Oral and Contact Toxicity to the Bumble Bee *Bombus terrestris* L. (Hymenoptera, Apidae) under Laboratory Conditions Report No. S21-06042 GLP Unpublished	N	UPL
KCP 5.1.2/06 (filed in KCP	T. Ansaloni	2022b	Prothioconazole/Sulphur (50+625) g/L SC: Honey Bee (*Apis mellifera* L.) Chronic Oral Toxicity Test (10-Day Feeding) under Laboratory Conditions	N	UPL

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
10.3.1.2/01)			Report No. S21-06044 GLP Unpublished		
KCP 5.1.2/07 (filed in KCP 10.3.1.3/01)	T. Ansaloni	2022c	Prothioconazole/Sulphur (50+625) g/L SC: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions Report No. S21-06046 GLP Unpublished	N	UPL
KCP 5.1.2/08 (filed in KCP 10.4.1.2/01)	T. Vollmer	2023	A Field Study to Evaluate the Effects of Metabolites of Prothioconazole on Earthworm Populations Report No. S21-03781 GLP Unpublished	N	UPL
KCP 5.1.2/09 (filed in KCP 10.6.2/01)	D. Ripperger	2022	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Effects on the Seedling Emergence and Seedling Growth of Terrestrial Plant Species UPL report No.: S21-05533 GLP Unpublished	N	UPL
KCP 5.1.2/10 (filed in KCP 10.6.1/01)	D. Ripperger	2023	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Effects on the Vegetative Vigour of Terrestrial Plant Species UPL report No.: S21-05534 GLP Unpublished	N	UPL
KCP 5.1.2/11 (filed in KCP 2.1)	V. Buchholz	2022	Physical and Chemical Properties in one batch of prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04) Initial tests Report No. R C1254 GLP Unpublished	N	UPL
KCP 5.2/01 (filed in KCP 5.1.2/01)	J. Pearson	2022	Prothioconazole-desthio: Method Validation in Crops Company Report No. QG/20/011 GLP Unpublished	N	UPL
KCP 5.2/02	N. Boubakri	2023	Independent Laboratory Validation of Multi Residue Method for Determination of Prothioconazole-desthio in Different Matrices of Plant Origin Report No. S21-08354 GLP	N	UPL

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
			Unpublished		
KCP 5.2/03	D. Kleinhenz	2023	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Food of Animal Origin Report No. S21-08355 GLP Unpublished	N	UPL
KCP 5.2/04	T. Rastogi	2023	Independent Laboratory Validation of Prothioconazole-desthio in Different Matrices of Animal Origin Report No. S21-08868 GLP Unpublished	N	UPL
KCP 5.2/05	J. Hitchens	2023	Prothioconazole-desthio and hydroxy metabolites: Method Validation in Honey Report No. QG/21/009 GLP Unpublished	N	UPL
KCP 5.2/06	J. Wanger	2023	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Honey Report No. S21-08357 GLP Unpublished	N	UPL
KCP 5.2/07	M. Kaiser	2022	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Soil Report No. S21-08358 GLP Unpublished	N	UPL
KCP 5.2/08	M. Kaiser	2022	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Water Report No. S21-08359 GLP Unpublished	N	UPL
KCP 5.2/09	S. Jooß	2023	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Water Report No. S21-08869 GLP Unpublished	N	UPL

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
KCP 5.2/10	N. Boubakri	2023	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Urine Report No, S21-08361 GLP Unpublished	N	UPL

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

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
KCP 5/KCA 4/01 (IIA 8.2.4/01)	F. Heimbach	1999	Acute toxicity of JAU 6476 (tech.) to water fleas (Daphnia magna) Bayer AG, Report No.: HBF/DM 212 GLP Unpublished	N	Bayer
KCP 5/KCA 4/02 (IIA 8.2.2.2/02)	[redacted]	2002	JAU 6476-desthio: Early life-stage toxicity test with rainbow trout (Oncorhynchus mykiss) under flowthrough conditions [redacted], Report No.: 1022.013.321 GLP Unpublished	Y	Bayer
KCP 5/ KCA 4/03 (IIA 4.2.4.1/01)	W. Maasfeld	2002a	Method for the determination of JAU 6476 in air by HPLC-MS/MS Bayer AG, Report No.: 00724 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/04 (IIA 4.2.4.1/02)	W. Maasfeld	2002b	Method for the determination of JAU 6476-desthio in air by HPLC-MS/MS Bayer AG, Report No.: 00731 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/05 (IIA 4.2.1.1/06)	R. D. Weeren and S Pelz	2000	Modification M033 of method 00086: Validation of DFG method S 19 (extended revision) for the determination of residues of JAU 6476-desthio in materials of plant and animal origin. Dr. Specht & Partner, Chemische Laboratorien GmbH, Hamburg, Germany Bayer AG, Report No.: 00086/M033	N	Bayer

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
			GLP Unpublished		
KCP 5/ KCA 4/06 (IIA 4.2.1.1/07)	Th. Class	2001	Independent laboratory validation of DFG method S19 (extended revision) for the determination of residues of JAU 6476-desthio (BAYER method 00086/M033) in plant materials PTRL Europe, Ulm, Germany Bayer AG, Report No.: P/B 484 G GLP Unpublished	N	Bayer
KCP 5/ KCA 4/07 (IIA 4.2.1.1/03)	O. Heinemann	2001a	Analytical determination of residues of JAU6476-sulfonic acid and JAU6476-desthio in/on cereals and canola by HPLC-MS/MS Bayer AG, Report No.: 00647, GLP Unpublished	N	Bayer
KCP 5/ KCA 4/08 (IIA 6.1.1.1/04)	M. Haas	2001	Extraction efficiency testing of the residue method (00647) for the determination of JAU 6476 residues in spring wheat using aged radioactive residues Bayer AG, Report No.: MR-084/01 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/09 (IIA 4.2.1.1/05)	O. Heinemann	2001b	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio, and JAU6476-desthio in milk by HPLC-MS/MS (00655/M001) Bayer AG, Report No.: 00655/M001 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/10 (IIA 4.2.1.1/04)	O. Heinemann	2001c	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio, and JAU6476-desthio in/on matrices of animal origin by HPLC-MS/MS Bayer AG, Report No.: 00655 GLP Unpublished	N	Bayer
(KCP 5/ KCA 4/11 (IIA 4.2.1.1/08)	L. Dubey	2001	Independent laboratory validation of bayer methods 00655 and 00655/M001 for the determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio, and JAU6476-desthio in/on matrices of animal origin by HPLC-MS/MS Battelle, Geneva Research Centres, Carouge/Geneva, Switzerland Bayer AG, Report No.: A-14-01-01 GLP Unpublished	N	Bayer

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/ KCA 4/12 (IIA 4.2.2.1/01)	O. Schramel	2000	Residue analytical method 00610 (MR-643/99) for the determination of JAU 6476 and the metabolites JAU6476-desthio and JAU6476-S-methyl in soil by HPLC-MS/MS Bayer AG, Report No.: 00610 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/13 (IIA 4.2.2.1/03)	S. Steinhauer	2001	Enforcement method 00086/M038 for the determination of the residues of JAU 6476-desthio in soil - validation of DFG method S 19 (extended revision) -Dr. Specht & Partner, Chemische Laboratorien GmbH, Hamburg, Germany Bayer AG, Report No.: 00086/M038 GLP Unpublished	N	Bayer
KCP 5/ KCA 4/14 (IIA 4.2.3.1/03)	H. Sommer	2001	Enforcement method 00684 for determination of JAU 6476 and JAU 6476-desthio in drinking and surface water by HPLC-MS/MS Bayer AG, Report No.: 00684 GLP Unpublished	N	Bayer

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 and sulphur

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

Comments of zRMS:	The method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole-desthio in crops (oilseed rape - seed and forage; wheat - grain, straw, forage; barley - grain, straw, forage; field bean (dried); orange (whole) and cucumber) with the LOQ of 0.005 mg/kg for prothioconazole-desthio. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% for all matrices at each level (0.005 and 0.05 mg/kg). The method is acceptable.

Reference:	KCP 5.1.2/01
Report	Prothioconazole-desthio: Method Validation in Crops, J. Pearson, 2022, Report No. QG/20/011
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole-desthio content in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of prothioconazole-desthio were extracted from crops with acetonitrile and QuEChERS extraction salts, followed by centrifugation. An aliquot of the acetonitrile phase was then cleaned up using d-SPE followed by centrifugation. An aliquot was diluted two-fold with acetonitrile:water (80:20, v/v) for oilseed rape seed; wheat – grain and straw; barley – grain and straw; and field bean (dried) matrices. An aliquot was diluted ten-fold with acetonitrile:water (80:10, v/v) for cucumber, orange (whole), wheat forage, barley forage and oilseed rape forage matrices. A portion of the final extract was then taken for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring two ion mass transitions, except for stability samples where the quantitation transition only was monitored.

Instrument Conditions
Liquid Chromatography:

Column:	Kinetex 5u XB-C18 100A, 150 x 4.6 mm, 5 µm		
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287		
Column Oven Temperature (°C):	40		
Injection Volume * (µL):	10		
Flow Rate (µL/min):	1000		
Mobile Phase A:	0.1 % Formic acid in water		
Mobile Phase B:	0.1 % Formic acid in methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	40	60
	1.00	40	60
	5.00	10	90
	6.00	10	90
	6.10	0	100
	7.00	0	100
	7.10	40	60
	9.00	40	60
Flow divert:	Time (minutes)	Position	
	0.0	To waste	
	4.0	To mass spec	
	6.0 - End	To waste	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)		
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)		

* Alternative volumes are allowed, based on instrument performance

Mass Spectrometry:

MS/MS System	API 5500
Ionisation Mode:	ESI
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	5500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10
Declustering Potential (DP)** (V)	126

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass*	Q3 Mass*	Dwell Time (msec)	Collision Energy* (CE) (V)	Collision Exit Potential* (CXP) (V)	Declustering Potential (DP)	Expected Retention time (±0.5mins)
Prothioconazole -desthio	1#	312.1	70.1	100	47	12	126	5.4
	2#	312.1	125.0	100	49	6	126	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

* Minor modifications are allowed to optimise instrument performance.

Results and discussions

The analytical method for the determination of prothioconazole-desthio content in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (< 30% LOQ) in reagent blank and duplicate control samples, demonstrating good selectivity of the method.

Linearity

Calibration curves were obtained from a minimum of eight calibration solutions containing

prothioconazole-desthio. The standard calibration range was 0.075 to 3.0 ng/mL for wheat – straw and forage; barley – straw and forage; and oilseed rape forage matrices and 0.150 – 6.0 ng/mL for orange (whole), cucumber, wheat grain, barley grain, oilseed rape seed and field bean (dried) matrices (equivalent to 0.0015 – 0.06 mg/kg), covering a range from 30% of the LOQ to 20% above the highest fortification level. Matrix matched standards were prepared. Correlation coefficients $r \geq 0.995$ were achieved in the validation and extract stability batches using a linear regression with a weighting, demonstrating acceptable linearity. On visual inspection of a plot of residuals, the data was randomly distributed, demonstrating suitability of the chosen function.

Accuracy and Precision

For each matrix, average recoveries and the overall average recovery at the LOQ fortification level were all within the acceptance range of 60-120% (0.005 mg/kg) and average recoveries and the overall average recovery at the 10 x LOQ fortification level were all within the acceptance range of 70-120% (0.05 mg/kg), demonstrating satisfactory accuracy. The relative standard deviation (RSD) at and the overall RSD did not exceed 30% at the LOQ level or 20% at the 10 x LOQ level, demonstrating satisfactory precision. In addition, LC-MS/MS monitoring two ion mass transitions is considered to be a highly specific technique.

Matrix Effects

Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions prepared at the same concentration. Experiments assessed whether or not matrix effects were significant (i.e. ≥20% enhancement or suppression). No significant matrix effects were observed for orange, cucumber, field bean (dried), oilseed rape – seed and forage; wheat forage or barley – forage and straw; matrices. Significant matrix effects were observed for wheat – grain and straw; and barley grain matrices.

Stability of Standards and Extracts

Prothioconazole-desthio was shown to be stable in wheat forage, barley straw and oilseed rape forage sample extracts for 3 days, in cucumber sample extracts for 4 days, in wheat straw, barley forage and oilseed rape seed matrices for 5 days, in orange (whole) and field bean (dried) sample extracts for 6 days and in wheat grain and barley grain sample extracts for 7 days when stored refrigerated. All samples were analysed within the defined stability periods. Stock standard solutions of prothioconazole-desthio prepared in acetonitrile were shown to be stable for up to 86 days when stored refrigerated. Intermediate standard solutions of prothioconazole-desthio prepared in acetonitrile:water (9:1, v/v) were shown to be stable for up to 71 days when stored refrigerated.

LOD and LOQ

The limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) for prothioconazole-desthio in crops, equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole-desthio in crops, as confirmed by recovery efficiency testing.

Prothioconazole-desthio accuracy and precision data in wheat grain

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	104	105	108	106	100	5	104	2.8	100-108
0.05	103	102	105	99	101	5	102	2.1	99-105
Overall						10	103	2.6	99-108
Confirmatory Transition 312.1→125.0 m/z									
0.005	106	100	103	105	109	5	104	3.4	100-109
0.05	104	103	105	100	103	5	103	2.0	100-105
Overall						10	104	2.7	100-109

Prothioconazole-desthio accuracy and precision data in wheat forage

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	100	101	107	104	99	5	102	3.2	99-107
0.05	102	92	87	95	95	5	94	6.0	87-102
Overall						10	98	6.1	87-107
Confirmatory Transition 312.1→125.0 m/z									
0.005	101	102	105	104	101	5	102	1.7	101-105
0.05	104	94	88	96	95	5	95	5.8	88-104
Overall						10	99	5.4	88-105

Prothioconazole-desthio accuracy and precision data in wheat straw

Fortification Level (mg/kg)	Recoveries (%)					Number of Replicates (n)	Mean Recovery (%)	Relative Standard Deviation (RSD %)	Recovery Range (%)
Quantitation Transition 312.1→70.1 m/z									
0.005	94	117	97	108	97	5	103	9.4	94-117
0.05	110	108	92	99	119	5	106	9.8	92-119
Overall						10	104	9.2	92-119
Confirmatory Transition 312.1→125.0 m/z									
0.005	96	126	111	124	103	5	112	11.6	96-126
0.05	108	110	90	99	119	5	105	10.3	90-119
Overall						10	109	10.9	90-126

Table A 1: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in crops

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (> 30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points
Calibration range	0.075 to 3.0 ng/mL for wheat – straw and forage 0.150 – 6.0 ng/mL for wheat - grain equivalent to 0.0015 – 0.06 mg/kg
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD: 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion

The validation criteria for the method used to determine prothioconazole-desthio in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of residues of prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crop matrices (oilseed rape - seed and forage; wheat - grain, straw and forage; barley - grain, straw and forage; field bean (dried); orange (whole) and cucumber) with the LOQ of 0.005

	mg/kg. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% for all matrices at each level (0.005 and 0.05 mg/kg). The method is acceptable.

Reference:	KCP 5.1.2/02
Report	Hydroxy Metabolites of prothioconazole-desthio: Method Validation in Crops, D. Giancola, 2023, Report No. QG/22/001
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Quantitative determination of prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4- hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

For all matrices, the extracts were evaporated to the aqueous remainder by rotary evaporation. The extracts were then adjusted to pH 3-4 using 1M HCl and incubated for hydrolysis at 80°C for one hour. After hydrolysis, the sample extracts were then adjusted to pH 7 using 1M sodium bicarbonate solution. Sample extracts were then cleaned up by liquid/liquid partition using cyclohexane/ethyl acetate (85:15, v/v). The organic layer was then evaporated to dryness under a stream of nitrogen. After reconstitution in acetonitrile and water, a portion of the final extract is taken for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS) monitoring two ion mass transitions.

Instrument Conditions

Liquid Chromatography:

Column:	Kromasil 5 µm C18 100A 4.6 × 250 mm			
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287			
Column Oven Temperature (°C):	40			
Injection Volume * (µL):	20			
Flow Rate (µL/min):	1000			
Mobile Phase A:	0.1 % Formic acid in water			
Mobile Phase B:	Methanol			
Gradient:	Time (minutes)	Flow Rate (µL/min)	% Mobile Phase A	% Mobile Phase B
	0.00	1000	60	40
	0.20	1000	33	67
	8.70	1000	33	67
	8.80	900	33	67
	10.30	900	5	95
	11.80	900	5	95
	12.00	900	60	40
	12.50	900	60	40
	13.50	900	60	40
Flow divert:	Time (minutes)		Position	
	0.00		To waste (B)	
	5.20		To mass spec (A)	
	9.70		To waste (B)	
	12.50		To mass spec (A)	
	13.40 to end		To waste (B)	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)			
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)			

*Alternative volumes are allowed, based on instrument performance

Mass Spectrometry:

MS/MS System	API 5500
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	3500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass^	Q3 Mass^	Dwell Time (msec)	Collision Energy^ (CE)	Collision Exit Potential^ (CXP)	Declustering Potential^ (DP)	Expected Retention time (±0.5mins)
Prothioconazole-α-hydroxy-desthio	1#	328.265	69.900	100	36.00	10.00	80.00	5.76 and 7.85
	2#	328.265	141.200	100	39.00	10.00	80.00	
Prothioconazole-3-hydroxy-desthio	1#	328.142	70.000	100	37.00	10.00	46.00	7.29
	2#	328.142	141.00	100	47.00	10.00	46.00	
Prothioconazole-4-hydroxy-desthio	1#	328.154	70.000	100	35.00	12.00	121.00	8.35
	2#	328.154	141.100	100	53.00	10.00	121.00	
Prothioconazole-5-hydroxy-desthio	1#	327.948	70.000	100	33.00	14.00	151.00	8.94
	2#	327.948	141.000	100	45.00	22.00	151.00	
Prothioconazole-6-hydroxy-desthio	1#	327.899	140.872	100	68.00	10.00	69.00	12.9
	2#	327.899	69.824	100	44.00	10.00	69.00	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

^ Minor modifications are allowed to optimise instrument performance.

Results and discussions

The analytical method for the determination of hydroxy metabolites of prothioconazole-desthio content in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

Chromatographic interferences at the retention time of prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5- hydroxy-desthio and prothioconazole-6-hydroxy-desthio were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating the selectivity of the method.

Linearity

Calibration curves were obtained from a minimum of eight calibration solutions containing prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4- hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio. The standard calibration range was 0.375 to 15.0 ng/mL for oilseed rape seed, field beans (dried), barley grain, barley forage, cucumber, orange, barley straw and wheat straw matrices and 0.075 – 3.0 ng/mL for oilseed rape forage, wheat forage and wheat grain (equivalent to 0.0015 – 0.06 mg/kg), covering a range from 30% of the LOQ to 20% above the highest fortification level. Matrix matched standards were prepared. Correlation coefficients $r \geq 0.995$ were achieved in the validation and extract stability batches using a linear regression or quadratic regression with a weighting. On visual inspection of a plot of relative residuals, the data was randomly distributed, demonstrating suitability of the chosen function.

Accuracy and Precision

For each matrix, average recoveries and the overall average recovery at the LOQ fortification level were all within the acceptance range of 60-120% (0.005 mg/kg) and average recoveries and the overall average recovery at the 10 x LOQ fortification level were all within the acceptance range of 70-120% (0.05 mg/kg), demonstrating satisfactory accuracy. The relative standard deviation (RSD) at and the overall RSD did not exceed 30% at the LOQ level or 20% at the 10 x LOQ level, demonstrating satisfactory precision. In addition, LC-MS/MS monitoring two ion mass transitions is considered to be a highly specific technique.

Satisfactory accuracy and precision data were achieved for both ion mass transitions monitored, demonstrating that either transition may be used for quantification and/or confirmation of residues.

Matrix Effects

Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). No significant matrix effects were observed except for:

- prothioconazole-6-hydroxy-desthio in barley forage, barley straw, wheat straw, and oilseed rape seed.
- prothioconazole-5-hydroxy-desthio in oilseed rape seed and prothioconazole-5- hydroxy-desthio (quantitation transition) in oilseed rape forage.
- prothioconazole-4-hydroxy-desthio (confirmatory transition) in field bean (dried) and prothioconazole-4-hydroxy-desthio (quantitation transition) in oilseed rape forage
- prothioconazole-3-hydroxy-desthio in oilseed rape forage

Wheat grain matrix effect experiment was performed in two different analytical runs. Data obtained for prothioconazole-5-hydroxy-desthio (confirmatory transition) show contradictory results and no final matrix effect assessment can be determined. For this reason, it is recommended to use matrix matched standards when analysing prothioconazole-5-hydroxy-desthio (confirmatory transition) in wheat grain.

Wheat straw matrix effect experiment was performed in two different analytical runs. Data obtained for prothioconazole-5-hydroxy-desthio show contradictory results and no final matrix effect assessment can be determined. For this reason, it is recommended to use matrix matched standards when analysing prothioconazole-5-hydroxy-desthio in wheat straw.

Stability of Standards and Extracts

Prothioconazole-α-hydroxy-desthio, Prothioconazole-3-hydroxy-desthio, Prothioconazole-4- hydroxy-desthio, Prothioconazole-5-hydroxy-desthio and Prothioconazole-6-hydroxy-desthio were shown to be stable in wheat forage sample extracts for 8 days; in field bean (dried) and barley grain sample extracts for 7 days; in oilseed rape forage for 6 days; in wheat straw, barley straw, orange and wheat grain sample extracts for 5 days when stored refrigerated.

Standard solution stability for prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio, prothioconazole-6-hydroxy-desthio and prothioconazole-α-hydroxy-desthio prepared in acetonitrile:water 80:20 (v/v) was established in study QG/21/009 for 54 days when stored at approximately 4°C.

LOD and LOQ

The limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) for prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops, equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole-α-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in crops, as confirmed by recovery efficiency testing.

Table A 2: Characteristics for the analytical method used for validation of hydroxy metabolites of prothioconazole-desthio residues in crops

	Hydroxy metabolites of prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points.
Calibration range	0.375 - 15.0 ng/mL for oilseed rape seed, field beans (dried), barley grain, barley forage, cucumber, orange, barley straw and wheat straw matrices and 0.075 – 3.0 ng/mL for oilseed rape forage, wheat forage and wheat grain (equivalent to 0.0015 – 0.06 mg/kg). $r \geq 0.995$

	Hydroxy metabolites of prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	LOQ = 0.005 mg/kg LOD = 0.0015 mg/kg

Conclusion
The validation criteria for the method used to determine prothioconazole-desthio in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The analytical method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of residues of triazole derived metabolites (Triazole alanine (TA) Triazole acetic acid (TAA), Triazole lactic acid (TLA) and 1,2,4-triazole (TRZ)) in crops (barley grain, barley plant, barley straw, cucumber, field bean, oilseed rape plant, oilseed rape seed, orange (whole), wheat grain, wheat plant and wheat straw) with the LOQ of 0.005 – 0.2 mg/kg. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% for all TDMs and for all matrices at each level. The method is acceptable.

Reference:	KCP 5.1.2/03
Report	Triazole Derived Metabolites (TDM's) (1,2,4-triazole, triazole acetic acid, triazole alanine and triazole lactic acid): Method Validation in Crops, B. Phipps, 2024, Report No. QG/20/012
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	Yes – please refer to the report for a complete list. In summary, the deviations are considered not to have an impact on the validity of the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of residues of triazole derived metabolites (Triazole alanine (TA) Triazole acetic acid (TAA), Triazole lactic acid (TLA) and 1,2,4-triazole (TRZ)) in crops was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of TA, TAA, TLA and TRZ were extracted from barley grain, barley plant, barley straw, cucumber, field bean, oilseed rape plant, oilseed rape seed, orange (whole), wheat grain, wheat plant and wheat straw with methanol: water (4:1 v/v) by homogenisation with ultra-turrax followed by filtration under vacuum through celite. This was followed by filtration through SPE, concentration and reconstitution in water. A portion of the final extract was then filtered, diluted and internal standard added.

Final determination was by liquid chromatography with tandem mass spectrometry (LC-MS/MS) with SelexION technology, monitoring two ion mass transitions (or a single ion transition but with separate HPLC conditions)*, except for stability samples where the quantitation transition only was monitored. The method was validated in terms of linearity (calibration), specificity (selectivity), accuracy (recovery), precision (repeatability), matrix effects and stability.

*The exception to this was for triazole alanine where three sets of ion data were generated for cucumber, barley grain, barley plant, barley straw, wheat grain, wheat plant, wheat straw. Any valid data generated for any of these 3 ions is reported.

Instrument Conditions

Liquid Chromatography:

Column:	Thermo Aquasil C18, 150 × 3.0mm, 3µm		
Guard Column:	C18, 4 x 3.0mm ID		
Column Oven Temperature (°C):	40		
Injection Volume * (µL):	20		
Flow Rate (µL/min):	1000		
Mobile Phase A:	0.2 % Acetic acid in water		
Mobile Phase B:	0.2 % Acetic acid in Methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	100	0
	2.00	100	0
	5.00	10	90
	5.50	10	90
	5.60	100	0
	7.00	100	0
Flow divert:	Time (minutes)	Position	
	0.0	To waste (A)	
	0.1	To mass spec (B)	
	3.0 – 7.0	To waste (A)	
Autosampler Wash Solvent 1 (Strong):	Methanol		
Autosampler Wash Solvent 2 (Weak):	Methanol:water (10:90 v/v)		
Autosampler wash method:	Post clean with solvent 1 (s):	2	
	Valve clean with solvent 1 (s):	3	
	Valve clean with solvent 2 (s):	3	

* Alternative volumes are allowed, based on instrument performance.

Mass Spectrometry:

DMS-MS/MS System:	API 6500 with SelexION
Ionisation Mode:	ESI
Ion Source:	Turbo Spray
Polarity:	Positive
Scan Type:	MRM
Ion Spray Voltage (IS) (V):**	5500
Collision Gas (CAD):**	Medium (-2.00)
Source Temperature (TEM) (°C):**	650
DMS Temperature (DT) (°C):	150 (low)
Curtain Gas (CUR):**	50.0
Ion Source Gas 1 (GS1):**	40
Ion Source Gas 2 (GS2):**	60
Entrance Potential (EP) (V):	10
Separation voltage (SV) V:	3000

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition #	Q1 Mass	Q3 Mass	Dwell Time (msec)	CE (V)*	CXP (V)*	DP*	COV*	DMO*
TA	1	157.0	70.0	100	19.00	6.00	6.00	-0.83	-9.95
TA_ISTD	1	162.0	75.0	100	17.00	10.00	66.00	0.00	0.97

* Minor modifications are allowed to optimise instrument performance.

Transition 1 (confirmatory) is to be used for sample quantification for all non IL-IS transitions.

Results and discussions

The analytical method for the determination of residues of triazole derived metabolites (Triazole alanine (TA) Triazole acetic acid (TAA), Triazole lactic acid (TLA) and 1,2,4-triazole (TRZ)) in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity
Chromatographic interferences were detected (>30% of the limit of quantitation) in the replicate control samples for some analytes and matrices, please refer to the study for further details. Where interferences >30% of the LOQ in the control samples were observed, the average control found value in mg/kg was subtracted from the recovery value in mg/kg. All reagent blank samples analysed were <30% LOQ. Selectivity was confirmed by the use of LC-MS/MS monitoring two ion mass transitions or a single ion mass transition using two significantly different HPLC columns. A minimum of one reagent blank and two untreated replicates were analysed within each set of validation data (for each matrix and analyte).

Linearity
Calibration curves were obtained from a minimum of five calibration solutions containing the relevant analytes for analysis. Mixed analyte standards were used where necessary. The standard calibration range was 0.3 to 50 ng/mL, covering a range from at least 30% of the LOQ to at least 20% above the highest concentration level.

Accuracy and Precision
Average recoveries and the RSD calculated at each fortification level were within the limits described in the table below; demonstrating satisfactory accuracy and precision.

Concentration level (mg/kg)	Range of mean recoveries (%)	Precision, RSD (%)
≤0.01	60-120	30
>0.01 - ≤ 0.1	70-120	20
>0.1 - ≤ 1.0	70-110	15
>1	70-110	10

Satisfactory accuracy and precision data were achieved for both ion mass transitions monitored (or single ion transitions via two HPLC columns), demonstrating that either transition may be used for quantitation and/or confirmation of residues.

Matrix Effects
Matrix effects were investigated comparing peak area and peak area ratios of solvent standard solutions to peak area and peak area ratios of matrix-matched standard solutions prepared at the same concentration. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). Matrix effects were investigated with and without the addition of internal standard. Varying degrees of matrix effects were observed without the presence of internal standard. No significant matrix effects were observed. (i.e. >20% enhancement or suppression) when the internal standard was present. Matrix effects were negated by the addition of internal standard as such solvent standards, which were used for all analysis.

Stability of Solutions
Stock solutions of each analyte prepared in solvent were stored in a refrigerator. After storage, an aliquot of the stock solutions were diluted and analysed against a freshly prepared standard. Working, intermediate and calibration solutions were also stored and tested for stability. Peak areas of the stored solutions were compared to freshly prepared solutions and they were deemed stable if the areas were within 10% of each other

LOD and LOQ
The limit of detection (LOD) was established at 30% LOQ for each analyte and matrix.

Table A 3: Characteristics for the analytical method used for validation of triazole derived metabolites residues in wheat

	TRZ	TAA	TA	TLA
Specificity	samples for some analytes and matrices, please refer to the study for further details. Where interferences >30% of the LOQ in the control samples were observed, the average control found value in mg/kg was subtracted from the recovery value in mg/kg.			
Calibration (type, number of data points)	Minimum of 5 calibration points.			
Calibration range	0.3 – 50 ng/mL (equivalent to 0.0012 – 0.2 mg/kg) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.0024 – 0.4 mg/kg) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.0024 – 0.4 mg/kg) $r \geq 0.995$	0.3 – 50 ng/mL (equivalent to 0.0012 – 0.2 mg/kg) $r \geq 0.995$
Assessment of matrix effects is presented	Yes			
Limit of determination/quantification	LOQ = 0.005 mg/kg (wheat grain, wheat plant and wheat straw) LOD = 0.0015 mg/kg (wheat grain, wheat plant and wheat straw)	LOQ = 0.01 mg/kg (wheat grain), 0.02 mg/kg (wheat plant) and 0.05 mg/kg (wheat straw) LOD = 0.003 mg/kg (wheat grain), 0.006 mg/kg (wheat plant) and 0.015 mg/kg (wheat straw)	LOQ = 0.01 mg/kg (wheat grain), 0.02 mg/kg (wheat plant) and 0.05 mg/kg (wheat straw) LOD = 0.003 mg/kg (wheat grain), 0.006 mg/kg (wheat plant) and 0.015 mg/kg (wheat straw)	LOQ = 0.005 mg/kg (wheat grain) and 0.05 mg/kg (wheat plant and wheat straw) LOD =0.0015 mg/kg (wheat grain) and 0.0015 mg/kg (wheat plant and wheat straw)

Conclusion
The validation criteria for the method used to determine triazole derived metabolites residues in crops has been met in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	An analytical method was successfully validated for determination of sulphur and prothioconazole in reconstituted water and algal media. The limit of quantification (LOQ) of the analytical method was 4.052 µg/L for prothioconazole in reconstituted water and alga media and 12.732 mg/L for sulphur in reconstituted water and 10.115 mg/L for sulphur in alga media. The method is fit for purpose.

Reference:	KCP 5.1.2/04
Report	Validation of the Analytical Method for Determination of Active Substance Concentration and Stability of Prothioconazole + Sulphur in Matrix, Following the Application of Prothioconazole/Sulphur (50 + 625) G/L SC, M. Patel, 2023, Report No. 228-2-13-29109
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The analysis was performed to determine the sulphur and prothioconazole concentration and stability of Prothioconazole/Sulphur (50 + 625) g/L in reconstituted water and algal media by using LC-MS/MS for prothioconazole and ICP-MS for sulphur analysis.

Instrument Parameters (ICP-MS)

Instrument:	ICP-MS (Agilent Technologies, 7800 with Mass Hunter Software)
RF power:	1550 W

RF Matching:	1.60 V
Peak Pattern:	3 points
Spray chamber temperature:	2 ºC
Gas Flow Rate	
Plasma:	15.0 L/minute
Nebulizer:	1.05 L/minute
Auxiliary:	0.90 L/minute

Instrument Parameters (LC-MS/MS)
Instrument: LC-MS/MS (API 4000 Mass Spectrometer Couple with Nexera X2 HPLC System)
Column: Water X-Select CSH Fluoro-Phenyl (150 x 4.6 mm (i.d.), 3.5 µm particle size)
Injection Volume: 20.0 µL
Flow Rate: 0.6 mL/minute
Cooler Temperature: 15 ºC
Column Temperature: 40 ºC
Mobile Phase: Acetonitrile (80%) and 5 mM Ammonium bicarbonate in Milli Q water (20%), v/v

Sample Preparation
Each sample of prothioconazole was made in a volume of 10 ml using the Algal media (1X AAP media) as a diluent. Prior to injection into the LC-MS/MS, samples were further mixed with acetonitrile (1:1) ratio.

Each sample of sulphur was made in a volume of 4 ml using 2% HNO_3 in the Algal media (1X AAP media) as a diluent. The samples were directly injected into the ICP-MS.

Results and discussions
Specificity
The specificity of the method for the determination of prothioconazole concentration in the matrix was studies by injecting solvent (acetonitrile), mobile phase, blank test media (reconstituted water (RW), and algal media (AM - 1X AAP media)), blank formulation lowest level of linearity solution, test item solution at LOQ level. Since there was no interference between peaks of prothioconazole in reference standard, the test item, blank formulation, and blank test media, the method was considered specific for the analyte.

The specificity of the method for the determination of sulphur concentration in the matrix was studies by injecting 2 % HNO_3 in AM, 2 % HNO_3 in RW, blank test media (reconstituted water, and algal media (1X AAP media)), blank formulation lowest level of linearity solution, test item solution at LOQ level. Since there was no interference between peaks of sulphur in reference standard, the test item, blank formulation, and blank test media, the method was considered specific for the analyte.

Linearity
Working solutions of prothioconazole at concentrations of 0.989, 1.977, 3.954, 7.907, 15.813, 31.626 and 63.251 µg/L in RW and AM were prepared. Response was generated over the concentration range of 0.989 – 63.251 µg/L. An r value of 0.9932 was achieved for RW and a r value of 0.9983 for AM.

Working solutions of sulphur at concentrations of 1.00, 5.02, 10.04, 15.06, 20.08, 25.10 and 30.12 mg/L in 2 % HNO_3 RW and 2 % HNO_3 AM were prepared. Response was generated over the concentration range of 1.00 – 30.12 mg/L. An r value of 0.99912 was achieved for RW and a r value of 0.9916 for AM.

Precision
The method precision was assessed by the 5-fold determination of prothioconazole content in the test material at 4.052 µL in RW. A mean recovered concentration of 4.232 µL was attained with a %RSD of 3.37. This was repeated in AM and a mean recovered concentration of 4.178 µL was attained with a %RSD of 4.68.

The method precision was assessed by the 5-fold determination of sulphur content in the test material at 12.732 mg/mL in RW. A mean recovered concentration of 11.584 mg/mL was attained with a %RSD of 3.99. This was repeated in AM at 10.115 mg/mL and a mean recovered concentration of 10.782 mg/mL

was attained with a %RSD of 7.22.

Accuracy

Method accuracy was investigated by spiking RW with prothioconazole at 4.052 and 3857.255 µg/L. Mean recoveries of 104.45 and 108.86 %, respectively, were attained. Method accuracy was investigated by spiking AM with prothioconazole at 4.052 and 1763.317 µg/L. Mean recoveries of 103.12 and 85.50 %, respectively, were attained.

Method accuracy was investigated by spiking RW with sulphur at 12.732 and 127.316 mg/mL. Mean recoveries of 90.99 and 100.55 %, respectively, were attained. Method accuracy was also investigated by spiking AM with prothioconazole at 10.115 and 101.148 mg/mL. Mean recoveries of 106.59 and 107.91 %, respectively, were attained.

Table A 4: Recovery results from method validation of prothioconazole and sulphur using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Reconstituted water (RW)	Prothioconazole	4.052 µg/L n = 5	104.45	3.37	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Reconstituted water (RW)	Prothioconazole	3857.255 n = 5	108.86	7.37	
algal media (AM)	Prothioconazole	4.052 µg/L n = 5	103.12	4.68	
algal media (AM)	Prothioconazole	1763.317 µg/L n = 5	85.50	5.04	
Reconstituted water (RW)	Sulphur	12.732 mg/mL n = 5	90.99	3.99	
Reconstituted water (RW)	Sulphur	127.316 mg/mL n = 5	100.55	5.32	
algal media (AM)	Sulphur	10.115 mg/mL n = 5	106.59	7.22	
algal media (AM)	Sulphur	101.148 mg/mL n = 5	107.91	6.63	

Table A 5: Characteristics for the analytical method used for validation of prothioconazole and sulphur residues in reconstituted water and alga media

	Prothioconazole in RW	Prothioconazole in AM	Sulphur in RW	Sulphur in AM
Specificity	Intereference from the solvent, mobile phase, blank matrix (reconstituted water and algal media), lowest level of linearity solution, test item solution at LOQ level, and blank formulation solutions did not exceed the 30% peak area of the target analyte at the LOQ level			
Calibration (type, number of data points)	r = 0.9932 y = 8.41e+003 x + 804 n = 7	r = 0.9983 y = 1.58e+004 x +2.55e+003 n = 7	r = 0.99912 y = 18404.0345x + 131696.3367 n = 7	r = of 0.9916 y = 17938.1254x + 66482.0233 n = 7
Calibration range	0.989 – 63.251 µg/L	0.989 – 63.251 µg/L	1.00 – 30.12 mg/L	1.00 – 30.12 mg/L
Assessment of matrix effects is presented	yes			
Limit of determination/quantification	LOD = 0.989 µg/L LOQ = 4.052 µg/L	LOD = 0.989 µg/L LOQ = 4.052 µg/L	LOD = 1.00 mg/L LOQ = 12.732 mg/L	LOD = 1.00 mg/L LOQ = 10.115 mg/L

Conclusion
The method for the analysis of FHO04 in RW and AM fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

Comments of zRMS:	An analytical method was validated for determination of sulphur and prothioconazole in 50% (w/v) aqueous sucrose solution (matrix 1) and deionised water containing 0.1% Triton X 100 (matrix 2). The limit of quantification (LOQ) of the analytical method was 15.7 mg/L for prothioconazole and 475 mg/L for sulphur in matrix 1, and 78.3 mg/L for PTZ and 1412 mg/L for sulphur in matrix 2. The method is fit for purpose.

Reference:	KCP 5.1.2/05
Report	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Acute Oral and Contact Toxicity to the Bumble Bee *Bombus terrestris* L. (Hymenoptera, Apidae) under Laboratory Conditions, D., Ripperger, 2022, Report No. S21-06042.
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Prothioconazole
Analysis was performed to determine the concentration of prothioconazole in 50 % (w/v) aqueous sucrose solution and deionised water containing 0.1 % Triton X 100 by HPLC-MS/MS.

Instrument Parameters (HPLC-MS/MS)

Chromatographic conditions				
HPLC system	Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP			
Column	Phenomenex Kinetex 2.6 µm Biphenyl 100A, 100 mm x 2.1 mm i.d., 2.6 µm mean particle size (No. 00D-4622-AN) with 2.1 mm C18 UHPLC guard column			
Column oven temperature	40 °C			
Injection volume	5 µL			
Mobile phases	Eluent A: Water + 0.1 % (v/v) acetic acid Eluent B: Acetonitrile			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.01	70	30	600
	1.00	70	30	600
	3.50	1	99	600
	4.50	1	99	600
	4.60	70	30	600

	6.00	70	30	600
Divert valve	2.0 min to 3.5 min to MS			
Retention time(s)	approx. 2.7 min for prothioconazole			
Mass spectrometric conditions				
MS system	SCIEX API 5500			
Ionisation type	Electrospray ionization (ESI)			
Polarity	Positive ion mode			
Scan type	Multiple Reaction Monitoring (MRM)			

Capillary voltage (IS)	3000 V (pos)	Ionspray turbo heater (TEM)	550 °C	
Curtain gas (CUR)	40 (arbitrary units)	Gas flow 1 (GS1)	60 (arbitrary units)	
Collision gas (CAD)	9 (arbitrary units)	Gas flow 2 (GS2)	60 (arbitrary units)	

Analyte monitored	Ion mass transition monitored [m/z]	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 189	81	10	27	10	100
	344 → 154	81	10	39	14	100

Sample Preparation

A stock solution at about 17020 mg/L (test item concentration) was prepared in demineralized water. From the stock solution, appropriate fortification solution at 4000 mg/L was prepared in demineralized water. They were used for fortification of the following recovery samples:

- 2000 mg test item/L in deionised water containing 0.1 % Triton X 100
- 400 mg/L in 50 % (w/v) aqueous sucrose solution.

For preparation of 220000 mg/L recovery samples 110 ± 1.10 mg were accurately weighed and dissolved in 50 % (w/v) aqueous sucrose solution.

For preparation of 700000 mg/L recovery samples 350 ± 3.50 mg were accurately weighed and dissolved in deionised water containing 0.1 % Triton X 100.

Sulphur

Analysis was performed to determine the concentration of sulphur in 50 % (w/v) aqueous sucrose solution (matrix 1) and deionised water containing 0.1 % Triton X 100 (matrix 2) by ICP-OES.

Instrument Parameters (ICP-OES)

ICP-OES system	Agilent 5110 VDV
Carrier Gas	Argon
Plasma Flow	12 L/min
Viewing Mode	Radial
Read Time	10 s

Pump Speed	12 rpm (rounds per minute)

Sample Preparation
A stock solution at about 1000 mg/L (test item concentration) was prepared in 5 % nitric acid. This solution was used as fortification solution of the following recovery samples:

- Matrix 1: 1012 mg test item/L and 201600 mg test item/L
- Matrix 2: 3006 mg test item/L and 603400 mg test item/L

For preparation of the high recovery level samples 60 mg ± 1.2% were accurately weighed onto the respective blank (330 mg for Matrix 1 and 100 mg for Matrix 2) and dissolved in 7.5 mL HNO3 + 2.5 mL HCl.

Results and discussions

Prothioconazole
Specificity
The analyte was determined in the final sample dilutions by use of HPLC-MS/MS detection. One MS/MS mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated 50% (w/v) aqueous sucrose solution and deionised water containing 0.1% Triton X 100 samples were analysed according to the method to investigate the presence of residue and/or background interference at the retention time of prothioconazole. The samples showed no significant interference (above 30% of LOQ) at the retention time of the analyte in 50% (w/v) aqueous sucrose solution and deionised water containing 0.1% Triton X 100, therefore showing that the method is highly specific.

Linearity
Working solutions of prothioconazole in the range of 0.9 – 7.0 ng/mL were prepared in 50% aqueous sucrose solution corresponding to a concentration range of 4.50 – 35.0 mg/L. A linear response was achieved with an r value of 0.9992.

Working solutions of prothioconazole in the range of 0.9 – 7.0 ng/mL were prepared in deionised water containing 0.1% Triton X 100, corresponding to a concentration range of 22.5 - 175 mg/L. A linear response was achieved with an r value of 0.9989.

Accuracy and Precision
The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated 50% (w/v) aqueous sucrose solution and deionised water containing 0.1% Triton X 100 and subsequent determination of the recoveries upon applying the test method.

The recoveries and the precision/repeatability for the 50% aqueous sucrose solution are presented in the table below.

Matrix	Test Item Fortification Level [mg/L]	Prothioconazole Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	RSD [%]	n
Mass Transition *m/z* 344 → 189 (Quantification)						
50% (w/v) aqueous sucrose solution	400	15.7	90 97 92 94 92	93	3	5
	220000	8610	103 98 100 90 94	97	5	5

The recoveries and the precision/repeatability for the deionised water containing 0.1% Triton X 100 solution are presented in the table below.

Matrix	Test Item Fortification Level [mg/L]	Prothioconazole Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	RSD [%]	n
Mass Transition m/z 344 → 189 (Quantification)						
Deionised water containing 0.1% Triton X 100	2000	78.3	101 97 93 102 103	99	4	5
	700000	27400	88 89 91 91 96	91	3	5

Sulphur

Specificity

The analyte was determined in the sample dilution by use of ICP-OES detection. One (1) wavelength was evaluated. A second wavelength was monitored for confirmation of peak identity but was not used for quantification of samples. Tap water for accompanying sample work up, for determination of (procedural) recoveries was available at the Test Site of the Analytical Phase. At least one (1) control sample per analytical set was analysed to investigate the residue level of the analyte and to check for any background interferences at the expected wavelength of the analyte. The blank values at the expected wavelength of the analyte of the control sample material that was used for determinations of the (procedural) recoveries did not exceed the LOD. Since blank peaks were not observed, blank correction was not necessary. Furthermore, at least one (1) reagent blank sample, which is a sample work up without matrix present, was conducted in the course of the analytical phase. Reagent blank values did not exceed LOD.

Linearity

Working solutions of sulphur in the range of 500 – 5000 ng/mL were prepared in Matrix 1 corresponding to a concentration range of 83 - 830 mg/L. A linear response was achieved with an r value of 0.99992.

Working solutions of prothioconazole in the range of 500 – 5000 ng/mL were prepared in Matrix 2, corresponding to a concentration range of 250 - 2500 mg/L. A linear response was achieved with an r value of ≥ 0.995.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated 50% (w/v) aqueous sucrose solution (Matrix 1) and deionised water containing 0.1% Triton X 100 (Matrix 2) and subsequent determination of the recoveries upon applying the test method.

The recoveries and the precision/repeatability for Matrix 1 are presented in the table below.

Matrix	Test Item Fortification Level [mg/L]	Sulphur Nominal [mg/L]	Mean Recovery [%]	RSD [%]	n
50% (w/v) aqueous sucrose solution (Matrix 1)	1012	475	103	2	5

	201600	94680	101	2	5

The recoveries and the precision/repeatability for Matrix 2 are presented in the table below.

Matrix	Test Item Fortification Level [mg/L]	Sulphur Nominal [mg/L]	Mean Recovery [%]	RSD [%]	n
Deionised water containing 0.1% Triton X 100 (Matrix 2)	3006	1412	103	3	5
	603400	283400	105	2	5

Table A 6: Characteristics for the analytical method used for validation of prothioconazole and sulphur residues in Matrix 1 and 2

	Prothioconazole in Matrix 1	Prothioconazole in Matrix 2	Sulphur in Matrix 1	Sulphur in Matrix 2
Specificity	No significant interferences at the retention time of analyte in any of the blank matrix tested (< LOD).			
	m/z 344 → 169 (quantification) m/z 344 → 154 (confirmatory)		181.972 nm (quantification) 180.669 nm (comfirmatory)	
Calibration (type, number of data points)	r = 0.9992 y = 8.95e+003 x + -1.08e+003 n = 7	r = 0.9989 y = 8.68e+003 x + -7.61 n = 7	r = 0.99992 y = 0.1998 x + 6.6362 n = 7	r = > 0.995 n = 7
Calibration range	0.9 – 7.0 ng/mL (corresponding to 4.50 – 35.0 mg/L)	0.9 – 7.0 ng/mL (corresponding to 22.5 - 175 mg/L)	500 - 5000 ng/mL (corresponding to 83 – 830 mg/L	500 - 5000 ng/mL (corresponding to 250 – 2500 mg/L
Assessment of matrix effects is presented	yes			
Limit of determination/quantification	LOD = 4.50 mg/L LOQ = 15.7 mg/L	LOD = 22.5 mg/L LOQ = 78.3 mg/L	LOD = 83.0 mg/L LOQ = 475 mg/L	LOD = 250 mg/L LOQ = 1412 mg/L

Conclusion

The method for the analysis of FHO04 fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

Comments of zRMS:	An analytical method was validated for determination of sulphur and prothioconazole in 50% (w/v) aqueous sucrose solution + 0.1% xanthan gum. The limit of quantification (LOQ) of the analytical method was 7.56 mg/kg for prothioconazole and 188 mg/L for sulphur. The method is fit for purpose.

Reference:	KCP 5.1.2/06
Report	Prothioconazole/Sulphur (50+625) g/L SC: Honey Bee (*Apis mellifera* L.)

	Chronic Oral Toxicity Test (10-Day Feeding) under Laboratory Conditions, T. Ansaloni, 2022, Report No. S21-06044
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	No

Materials and methods

Prothioconazole

Analysis was performed to determine the concentration of prothioconazole in 50% (w/v) aqueous sucrose solution + 0.1% xanthan gum by LC-MS/MS.

Instrument Parameters (LC-MS/MS)

Chromatographic conditions				
HPLC system	Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP			
Column	Phenomenex Kinetex 2.6 µm Biphenyl 100A, 100 mm x 2.1 mm i.d., 2.6 µm mean particle size (No. 00D-4622-AN) with 2.1 mm C18 UHPLC guard column			
Column oven temperature	40 °C			
Injection volume	5 µL			
Mobile phases	Eluent A: Water + 0.1 % (v/v) acetic acid Eluent B: Acetonitrile			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.00	70	30	600
	1.00	70	30	600
	3.50	1	99	600
	4.50	1	99	600
	4.60	70	30	600
	6.00	70	30	600
Divert valve	2.0 min to 3.2 min to MS			
Retention time(s)	approx. 2.8 min for prothioconazole			
Mass spectrometric conditions				
MS system	SCIEX API 5500			
Ionisation type	Electrospray ionization (ESI)			
Polarity	Positive ion mode			

Scan type	Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	3000 V (pos)		Ionspray turbo heater (TEM)		550 °C	
Curtain gas (CUR)	40 (arbitrary units)		Gas flow 1 (GS1)		60 (arbitrary units)	
Collision gas (CAD)	9 (arbitrary units)		Gas flow 2 (GS2)		60 (arbitrary units)	
Analyte monitored	Ion mass transition monitored [m/z]	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 189	81	10	27	10	100
	344 → 154	81	10	39	14	100

Sample Preparation
About 10.5 mg of test item was dissolved in acetonitrile then diluted to be within the range of the calibration curve with acetonitrile/water (1:1, v/v).

Sulphur
Analysis was performed to determine the concentration of prothioconazole and sulphur in 50% (w/v) aqueous sucrose solution + 0.1% xanthan gum by ICP-OES.

Instrument Parameters (ICP-OES)

ICP-OES system	Agilent 5110 VDV
Carrier Gas	Argon
Plasma Flow	12 L/min
Viewing Mode	Radial
Read Time	10 s
Pump Speed	12 rpm (rounds per minute)

Sample Preparation
About 0.5 g of the test item were digested in a microwave with 7.5 mL HNO_3 + 2.5 mL HCl and filled up to 50 mL with ultra-pure water). Samples were diluted to be within the range of the calibration curve with 1.5 % HNO3 + 0.5 % HCl.

Results and discussions
Prothioconazole

Specificity
The analyte was determined in the sample dilution by LC-MS/MS detection. One mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. At least one control sample per analytical set was analysed to investigate the residue level of the analyte and to check for any background interferences at the expected retention time of the analyte. The blank values at the expected retention time of the analyte of the control sample material that was used for determinations of the (procedural) recoveries did not exceed the LOD. Since blank peaks were not observed, blank correction was not necessary. Furthermore, at least one reagent blank sample, which is a sample work up without matrix present, was conducted in the course of the analytical phase. Reagent blank values did not exceed the LOD.

Linearity
Working solutions of prothioconazole in the range of 0.9 – 7 ng/mL were prepared, corresponding to a concentration range of 7.56 – 58.8 mg/kg. A linear response was achieved with an r value of 0.9991.

Accuracy and Precision
The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated 50% (w/v) aqueous sucrose solution + 0.1% xanthan gum and subsequent determination of the recoveries upon applying the test method.

Sulphur
Specificity
The analyte was determined in the sample dilution by use of ICP-OES detection. Significant interferences at the retention time of analyte in any of the blank matrix tested (< LOD) was found in one analytical control sample. Therefore, the retain sample was analysed and no significant interferences at the retention time of analyte (< LOD) was found.
- Quantifier wavelength 181.972 nm (evaluated and used for quantification)
- Qualifier wavelength 180.669 nm (monitored for confirmation of peak identity but was not used for quantification)

Linearity
Working solutions of sulphur in the range of 70 - 7000 ng/mL were prepared in 50% (w/v) aqueous sucrose solution + 0.1% xanthan gum, corresponding to a concentration range of 34.7 - 3471 mg/kg. A linear response was achieved with an r value of ≥ 0.995

Accuracy and Precision
The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated 50 % (w/v) aqueous sucrose solution + 0.1 % xanthan gum and subsequent determination of the recoveries upon applying the test method.

Table A 7: Recovery results from method validation of sulphur using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
50% (w/v) aqueous sucrose solution + 0.1% xanthan gum	Prothioconazole	27.4 n=5	93	4	All mean recoveries were within the acceptable range of 70 – 120% with RSD ≤ 20%.
	Prothioconazole	2700 n = 5	97	3	
	Sulphur	190 n = 3	104	6	
	Sulphur	100 n=3	101	1	

Table A 8: Characteristics for the analytical method used for validation of sulphur residues in matrix

	Prothioconazole	Sulphur
Specificity	The analyte was determined in the sample dilution by use of LC-MS/MS detection. The blank values at the expected retention time of the analyte of the control sample material that was used for determinations of the (procedural) recoveries did not exceed the LOD. Furthermore, at least one reagent blank sample, which is a sample work up without matrix present, was conducted in the course of the analytical phase. Reagent blank values did not exceed the LOD. *m/z* 344 → 189 (quantification)	The analyte was determined in the sample dilution by use of ICP-OES detection. Significant interferences at the retention time of analyte in any of the blank matrix tested (< LOD) was found in one analytical control sample. Therefore, the retain sample was analysed and no significant interferences at the retention time of analyte (< LOD) was found.

	Prothioconazole	Sulphur
	m/z 344 → 154 (confirmatory)	
Calibration (type, number of data points)	r = 0.9991 y = 1.68e+004 x + -642 n = 7	r ≥ 0.995 y = 0.20002305x + 7.33826874 n = 7
Calibration range	0.9 – 7.0 ng/mL (corresponding to 7.56 – 58.8 mg/kg)	70 - 7000 ng/mL (corresponding to 34.7 - 3471 mg/kg)
Assessment of matrix effects is presented	yes	yes
Limit of determination/quantification	LOD = 27.4 mg/kg LOQ = 7.56 mg/kg	LOD = 34.7 mg/kg LOQ = 188 mg/L

Conclusion
The method for the analysis of FHO04 fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

Comments of zRMS:	An analytical method was successfully validated for determination of prothioconazole in larval diet with LOQ of 0.0783 mg/kg. For sulphur the analysis does not fulfil the requirements SANTE/2020/12830 rev. 1 due to the high natural background of sulphur in the larval diet. The method is fit for purpose for ptohioconazole.

Reference:	KCP 5.1.2/07
Report	Prothioconazole/Sulphur (50+625) g/L SC: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions, T. Ansaloni, 2022, Report No. S21-06046
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes (for prothioconazole analysis only)

Materials and methods
Prothioconazole
The objective of this study was to analyse samples of larval diet for residues of prothioconazole by LC-MS/MS and undertake a full method validation in accordance to guidance document SANTE/2020/12830, rev. 1.

Instrument Parameters (LC-MS/MS)

Chromatographic conditions for Prothioconazole in Larval diet	
HPLC system	Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)
Column	Phenomenex Kinetex 2.6 µm Biphenyl 100A, 100 mm x 2.1 mm i.d., 2.6 µm mean particle size (No. 00D-4622-AN)
Column oven temperature	40 °C
Injection volume	5 µL
Mobile phases	Eluent A: Water + 0.1 % Acetic acid Eluent B: Acetonitrile (pure)

Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]		
	0.0	70	30	600		
	1.0	70	30	600		
	3.5	1	99	600		
	4.5	1	99	600		
	4.6	70	30	600		
	6.0	70	30	600		
Divert valve	0.0 min to 2.0 min to waste; 2.0 min to 3.2 min to MS; 3.2 min to 6.0 min to waste					
Retention time	approx. 2.7 min					
Mass spectrometric conditions for Prothioconazole						
MS system	SCIEX API 5500					
Ionisation type	Electrospray ionisation (ESI)					
Polarity	Positive ion mode					
Scan type	Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	3000 V		Ionspray turbo heater (TEM)		550 °C	
Curtain gas (CUR)	Nitrogen set at 40 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 60 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 60 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 189	81	10	27	10	100
	344 → 154	81	10	39	14	100

Sample Preparation

Stock solutions of the test item (concentrations about 14860 mg/L and 4200 mg/L) were prepared by dissolving a weight of the test item with deionized water. From stock solution 14860 mg/L further dilutions of 4503 mg/L and 20 mg/L were prepared in deionized water. Solutions 4200 mg/L and 4503 mg/L were used for fortification of larval diet high level recovery samples and storage stability samples (450 mg test item/kg). The solution 20 mg/L was used for fortification of larval diet LOQ recovery samples (2 mg test item/kg).

Sulphur

The objective of this study was to analyse samples of larval diet for residues of sulphur by ICP-OES and undertake a full method validation in accordance to guidance document SANTE/2020/12830, rev. 1.

Instrument Parameters (ICP-OES)

ICP-OES system	Agilent 5110 VDV
Carrier Gas	Argon
Plasma Flow	12 L/min

Viewing Mode	Radial
Read Time	10 s
Pump Speed	12 rpm (rounds per minute)

Sample Preparation
Stock solutions of the test item (concentrations of 1000000 and 800000 µL) were prepared by dissolving a weight of the test item in 1.5 % HNO_3 + 0.5 % HCl. Further solutions were prepared via dilution of the stock solution with the same diluent.

Results and discussions

Prothioconazole

Specificity
The analyte was determined in the final sample dilutions by use of LC-MS/MS detection. One mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated larval diet for accompanying control sample work up, for determination of (procedural) recoveries and for preparation of matrix-matched calibration standards was available at the laboratory. At least one control sample per analytical set was analysed to investigate the residue level of the analyte and to check for any background interferences at the expected retention time of the analyte. The blank values at the expected retention times of the analyte of the control sample material that was used for determinations of the (procedural) recoveries did not exceed the LOD. Furthermore, at least one (1) reagent blank sample, which is a sample work up without larval diet present, was conducted in the course of the analytical phase. Reagent blank values did not exceed the LOD.

Linearity
The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.5 - 3.5 ng/mL. This range corresponds to a fortification level of 0.0206 - 0.145 mg/kg and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest prothioconazole concentration detected in any diluted sample extract. A linear response was achieved with an r value of 0.9995.

Accuracy and Precision
The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated larval diet and subsequent determination of the recoveries upon applying the test method.

Sulphur

Specificity
Significant interferences (> LOQ) at the retention time of analyte in the blank matrix were subtracted from the samples.
- Quantifier wavelength 181.972 nm (evaluated and used for quantification)
- Qualifier wavelength 180.669 nm (evaluated, used for qualification)

Linearity
The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at 9 concentration levels ranging from 60 - 20000 ng/mL. This range corresponds to a fortification level of 6 – 2000 mg/kg. A linear response was achieved with an r value of 0.9999.

Accuracy and Precision
The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated larval diet and subsequent determination of the recoveries upon applying the test method.

Table A 9: Recovery results from method validation of prothioconazole and sulphur using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Larval diet	Prothioconazole	17.6 mg/kg n = 3	94	7	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Larval diet	Sulphur	94.5 mg/kg n = 5	120	18	
Larval diet	Sulphur	189 mg/kg n = 5	129	11	

A mean recovery of 129% was attained at the 189 mg/kg fortification level and is therefore outside of the acceptable range. This is due to the high level of natural background of sulphur in larval diet. However, the purpose of the study was to determine the effect of repeated exposure of FHO04 to honey bee larvae. Since prothioconazole and sulphur were found to be stable, the determination of prothioconazole would allow for the consequent accurate determination of FHO04 and therefore sulphur. In the study, the concentration of FHO04 was determined by measuring the concentration of prothioconazole, not sulphur, and so the fact that the concentration of sulphur in the study could not be accurately determined would have no bearing on the results.

Table A 10: Characteristics for the analytical method used for validation of prothioconazole and sulphur residues in larval diet

	Prothioconazole	Sulphur
Specificity	The blank values at the expected retention times of the analyte of the control sample material did not ex-ceed the LOD. Furthermore, at least one reagent blank sample, which is a sample work up without larval diet present, was conducted in the course of the analytical phase. Reagent blank values did not exceed the LOD.	Significant interferences (> 30% LOQ) at the retention time of analyte in the blank matrix were subtracted from the samples.
Calibration (type, number of data points)	r = 0.9995 y = 1.1e+004 x + -1.93e+003 n = 7	r = 0.9999 y = 0.2096x + 5.821 n = 9
Calibration range	0.5 - 3.5 ng/mL (corresponding to 0.0206 - 0.145 mg/kg)	6 – 20000 ng/L (correspnding to 6 – 2000 mg/kg)
Assessment of matrix effects is presented	yes	yes
Limit of determination/quantification	LOD = 0.0206 mg/kg LOQ = 0.0783 mg/kg	LOD = 6 mg/kg LOQ = 95.5 mg/kg

Conclusion

The method for the analysis of FHO04 in larval diet fulfils the requirements of SANTE/2020/12830 rev. 1 for prothioconazole. The analysis of FHO04 in larval diet does not fulfil the requirements SANTE/2020/12830 rev. 1 due to the high natural background of sulphur in the larval diet.

Comments of zRMS:	An analytical method was successfully validated for determination of prothioconazole, prothioconazole- desthio and s-methyl-prothioconazole in soil samples with LOQ of 0.01 mg/kg. The method is fit for purpose.

Reference:	KCP 5.1.2/08
Report	A Field Study to Evaluate the Effects of Metabolites of Prothioconazole on

	Earthworm Populations, T. Vollmer, 2023, Report No. S21-03781
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

The objective of this study was to analyse samples of soil for residues of prothioconazole, prothioconazole-desthio and s-methyl-prothioconazole and to analyse samples of spray solutions and petri dish samples for residues of prothioconazole.

Instrument Parameters (LC-MS/MS)

Chromatographic conditions for prothioconazole, prothioconazole-desthio and prothioconazole-s-methyl				
HPLC system	1290 Infinity HPLC System, Agilent Technologies			
Pre-column	SecurityGuard™ ULTRA for c18 UHPLC (2.1 mm, Phenomenex, Art. No. AJO-9782)			
Column	Agilent ZORBAX Eclipse XDB-C18, 600 bar, 50 mm x 4.6 mm, 1.8 µm			
Column oven temperature	40 °C			
Injection volume	40 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate in 0.1 % formic acid (v/v) Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	80	20	400
	0.30	80	20	400
	4.00	10	90	400
	6.50	10	90	400
	6.51	80	20	400
	8.50	80	20	400
Divert valve	0.0 min to 5.5 min to waste; 5.5 min to 8.0 min to MS; 8.0 min to 8.5 min to waste			
Retention time	Prothioconazole: approx. 5.9 min Prothioconazole-desthio: approx. 5.7 min Prothioconazole-s-methyl: approx. 6.7 min			
Mass spectrometric conditions for prothioconazole, prothioconazole-desthio and prothioconazole-s-methyl				
MS system	SCIEX API 6500 System, SCIEX (Triple quadrupole mass spectrometer			
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)			
Polarity	Positive ion mode			
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)			
Capillary voltage (IS)	4500 V (pos)	Ionspray turbo heater (TEM)	450 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)	Gas flow 1 (GS1)	Nitrogen set at 50 (arbitrary units)	

Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 154	66	10	43	16	50
	344 → 189	66	10	29	22	50
Prothioconazole-desthio	312 → 125	101	10	45	14	50
	312 → 70	101	10	59	10	50
Prothioconazole-s-methyl	358 → 116	50	10	30	20	50
	358 → 89	50	10	70	10	50

Results and discussions

Specificity

The analyte was determined in the final sample dilutions by use of LC-MS/MS detection. One mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated sample for accompanying control sample work up, for determination of (concurrent) recoveries and for preparation of matrix-matched calibration standards originated from the current study. At least two control samples per analytical set was analysed to investigate the residue level of the analyte and to check for any background interferences at the expected retention time of the analytes. The blank values at the expected retention times of the analytes of the control sample material that was used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ. Furthermore, at least one (1) reagent blank sample, which is a sample work up without matrix present, was conducted in the course of the analytical phase. Reagent blank values did not exceed 30 % of the LOQ.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.15 – 10.0 ng/mL. This range corresponds to a fortification level of 0.003 - 0.2 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any diluted sample extract. A linear response was achieved with an r value of ≥ 0.99.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated test portions and respective matrix and subsequent determination of the recoveries upon applying the test method.

Table A 11: Recovery results from method validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg)	Mean recovery (%)	RSD (%)
Prothioconazole (m/z 344 → 154)				
Soil	0.01	90, 94	92	2
	0.1	-	-	0
	1	107, 100, 97, 89, 103	99	5
Prothioconazole-desthio (m/z 312 → 125)				
Soil	0.01	113, 110, 112, 111, 119, 117	114	3
	0.1	97, 99, 106, 113	107	7

Matrix	Analyte	Fortification level (mg/kg)	Mean recovery (%)	RSD (%)
	1	104, 99, 99, 93, 103	100	4
Prothioconazole-s-methyl (m/z 358 → 116)				
Soil	0.01	114, 113, 111, 112, 123, 117	115	4
	0.1	96, 98, 106, 112	103	7
	1	106, 103, 103 97, 106	103	4

Table A 12: Characteristics for the analytical method used for validation of prothioconazole, prothioconazole-desthio and s-methyl-prothioconazole in soil

	Prothioconazole, prothioconazole-desthio and s-methyl-prothioconazole
Specificity	Blank values at the exprected retention times of the analytes of the control sample material did not exceed 30% of the LOQ.
Calibration (type, number of data points)	r ≥ 0.99 n = 5
Calibration range	0.15 – 10.0 ng/mL (corresponding to 0.003 - 0.2 mg/kg)
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD = 0.003 mg/kg LOQ = 0.01 mg/kg

Conclusion
The method for the analysis for the determination of prothioconazole, prothioconazole-desthio and s-methyl-prothioconazole in soil fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

Comments of zRMS:	Analytical methods for the determination of sulphur and prothioconazole in tap water were validated with regard to recovery, linearity of detector response, repeatability, specificity, matrix effect, stability of working solutions, limit of quantification and limit of detection. The analytical methods fulfil the requirements of guideline SANTE/2020/12830 rev. 1.

Reference:	KCP 5.1.2/09
Report	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Effects on the Seedling Emergence and Seedling Growth of Terrestrial Plant Species, D. Ripperger, 2022, Report No. S21-05533
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Prothioconazole
The objective of this study was to analyse samples of tap water for residues of prothioconazole by HPLC-MS/MS and undertake a full method validation in accordance to guidance document SANTE/2020/12830, rev. 1.

Instrument Parameters (HPLC-MS/MS)

Chromatographic conditions	
HPLC system	Agilent Technologies 1100 Infinity

Column	Kinetex® 2.6 µm C18 100 Å, 50 x 4.6 mm, 2.6 µm, Phenomenex			
Pre Column	HPLC guard column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)			
Column oven temperature	30 °C			
Injection volume	10 µL			
Mobile phases	Eluent A: Acetonitrile Eluent B: Water + 0.5% Formic Acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	75	25	500
	3.5	75	25	500
Divert valve	1.0 to 2.2 to MS			
Retention time	approx. 1.7 min for Prothioconazole			

Mass spectrometric conditions						
MS system	SCIEX API 4000					
Ionisation type	Electrospray ionization (ESI)					
Polarity	Positive mode					
Scan type	Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)		Ionspray turbo heater (TEM)		400 °C	
Curtain gas (CUR)	30 (arbitrary units)		Gas flow 1 (GS1)		50 (arbitrary units)	
Collision gas (CAD)	12 (arbitrary units)		Gas flow 2 (GS2)		50 (arbitrary units)	
Analyte monitored	Ion mass transition monitored [m/z]	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 o 326*	41	10	15	22	200
	344 o 189	41	10	29	12	200

Sample Preparation

Stock solutions of the prothioconazole (concentrations of 0.5 – 50 ng/mL) were prepared by dissolving a weight of the test item in tap water. Further solutions were prepared via dilution of the stock solution with the same diluent.

Sulphur

The objective of this study was to analyse samples of tap water for residues of Sulphur by ICP-OES and undertake a full method validation in accordance to guidance document SANTE/2020/12830, rev. 1.

Instrument Parameters (ICP-OES)

ICP-OES system	Agilent 5110 VDV
Carrier Gas	Argon
Plasma Flow	12 L/min
Viewing Mode	Radial
Read Time	10 s
Pump Speed	12 rpm (rounds per minute)

Sample Preparation

Stock solutions of the sulphur (concentrations of 500 – 15000 ng/mL) were prepared by dissolving a weight of the test item in tap water. Further solutions were prepared via dilution of the stock solution with the same diluent.

Results and discussions

Prothioconazole

Specificity

For prothioconazole, one MS/MS mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. Untreated tap water samples were analysed according to the method to investigate the presence of residue and/or background interference at the retention time/s of prothioconazole. The samples showed no significant interference (above 30 % of LOQ) at the retention time/s of the analyte/s in tap water, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five concentration levels ranging from 0.5 – 50.0 ng/mL. This range corresponds to a fortification level of 50 - 5000 mg/L. A linear response was achieved with an r value of 0.9997.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated tap water and subsequent determination of the recoveries upon applying the test method.

Sulphur

Specificity

For sulphur, one selected elemental wavelength was evaluated. One additional wavelength was monitored for confirmation of peak identity but were not used for quantification. Untreated tap water samples were analysed according to the method to investigate the presence of residue and/or background interference at the retention time/s of prothioconazole. The samples showed no significant interference (above 30 % of LOQ) at the retention time/s of the analyte/s in tap water, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five concentration levels ranging from 500 - 15000 ng/mL. This range corresponds to a fortification level of 250 - 7500 mg/L. A linear response was achieved with an r value of 0.9993.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated

tap water and subsequent determination of the recoveries upon applying the test method.

Table A 13: Recovery results from method validation of prothioconazole and sulphur using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Tap water	Prothioconazole	392 mg/L n = 5	107	1	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Tap water	Prothioconazole	5093 mg/L n = 5	98	2	
Tap water	Sulphur	4710 mg/L n = 5	97	2	
Tap water	Sulphur	61190 mg/L n = 5	99	2	

Table A 14: Characteristics for the analytical method used for validation of prothioconazole and sulphur residues in tap water

	Prothioconazole	Sulphur
Specificity	The samples showed no significant interference (above 30 % of LOQ) at the retention time/s of the analyte/s in tap water, therefore showing that the method is highly specific.	
Calibration (type, number of data points)	r = 0.9997 2.59e+004 x + -3.8e+003 n = 6	r = 0.9993 y = 0.2096x +4.525 n = 7
Calibration range	0.5 – 50.0 ng/mL (corresponding to 50 - 5000 mg/L)	500 - 15000 ng/mL (corresponding to 250 - 7500 mg/L)
Assessment of matrix effects is presented	yes	yes
Limit of determination/quantification	LOD = 50 mg/L LOQ = 392 mg/L	LOD = 250 mg/L LOQ = 4710 mg/L

Conclusion

The method for the analysis of FHO04 fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

Comments of zRMS:	Analytical methods for the determination of sulphur and prothioconazole in tap water were fully validated in EAS-Study S21-05533 (RIPPERGER, 2022). A reduced validation with regard to recovery, linearity of detector response, repeatability, specificity, stability of working solutions, limit of quantification and limit of detection was performed within this study. The analytical methods fulfil the requirements of guideline SANTE/2020/12830 rev. 1.

Reference:	KCP 5.1.2/10
Report	Prothioconazole/Sulphur (50+625) g/L SC (FHO04): Effects on the Vegetative Vigour of Terrestrial Plant Species, D. Ripperger, 2023, Report No. S21-05534
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

The analytic methods for the determination of prothioconazole and sulphur in this study are identical to those used in KCP 5.1.2/09 (D. Ripperger, 2022, Report No. S21-05533), and have been fully validated in accordance with SANTE/2020/12830 rev. 1.

Comments of zRMS:	The analytical method for the determination of prothioconazole and sulphur was fully validated according to SANCO/3030/99 rev. 5 and fulfils the requirements of SANTE/2020/12830 rev. 1.

Reference:	KCP 5.1.2/11
Report	Physical and Chemical Properties in one batch on Prothioconazole/Sulphur (50+625) g/L SC (Formulation code FHO04) Initial tests, V. Buchholz, 2022, Report No. C1254
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole and sulphur in FHO04 was determined by HPLC-PDA in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions
Prothioconazole
Column: Syncronis C18, 4.6 x 250 mm, 5 µm particle size (Thermo reference : 97105-254630)
Elution:
Mobile phase A: Water T1 + 0.1% phosphoric acid Add 1000 mL of water T1 + 1 mL of phosphoric acid. Place the flask in an ultrasonic bath for 5 minutes.
Mobile phase B: Acetonitrile + 0.1% phosphoric acid Add 1000 mL of acetonitrile + 1 mL of phosphoric acid. Place the flask in an ultrasonic bath for 5 minutes.
Column temperature: 40°C
Detection: PDA at 258 nm
Volume injected: 20 µL
Retention time: Prothioconazole: ~ 18.3 min

Sulphur
Column: Zorbax Eclipse XDB-C18, 4.6 x 150 mm, 5 µm particle size (Agilent reference: 993967-902)
Elution:
Mobile phase A: Water T1
Mobile phase B: Acetonitrile
Column temperature: 25°C
Detection: PDA at 215 nm
Volume injected: 20 µL
Retention time: Sulphur: ~ 11.0 min

Results and discussions
Linearity
The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of seven concentration levels ranging from 2.5 – 50.0 µg/mL. This range corresponds to a fortification level of 50 - 5000 mg/L. A linear response was achieved with an r value of 0.99996 for prothioconazole and 0.99997 for sulphur.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated tap water and subsequent determination of the recoveries upon applying the test method.

Table A 15: Recovery results from method validation of prothioconazole and sulphur using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%)	RSD (%)	Comments
Blank formulation	Prothioconazole	5 µg/ml n = 5	94.8	0.51	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Blank formulation	Prothioconazole	40 µg/ml n = 5	99.6	1.72	
Blank formulation	Sulphur	5 µg/ml n = 5	100.5	0.76	
Blank formulation	Sulphur	40 µg/ml n = 5	96.9	0.5	

Table A 16: Characteristics for the analytical method used for validation of prothioconazole and sulphur

	Prothioconazole	Sulphur
Specificity	The specificity of the method was evaluated by the absence of interfering peaks in the area of interest when injecting solvent and blank formulation solution analysis. No interference can be seen in the area of analyte retention time during the analysis of the solvent and blank formulation	
Calibration (type, number of data points)	r = 0.9996 n = 7	r = 0.9997 n = 7
Calibration range	2.5 – 50.0 µg/mL	2.5 – 50.0 µg/mL
Assessment of matrix effects is presented	yes	yes
Limit of determination/quantification	LOD = 0.2 µg/mL LOQ = 5 µg/mL	LOD = 0.3 µg/mL LOQ = 5 µg/mL

Conclusion
The method for the analysis of prothioconazole and sulphur in FHO04 fulfils the requirements of SANTE/2020/12830 rev. 1 and has shown to be valid.

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)

Independent laboratory validation

Comments of zRMS:	The analytical method QG/20/011 was successfully independently validated for the determination of residues of prothioconazole-desthio in different plant matrices (high water content, high acid, high oil content and high protein/high starch content) with LOQ of 0.005 mg/kg. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% for all analytes at each level (0.005 and 0.05 mg/kg). The acceptance criteria of the SANTE/2020/12830 rev.1 for the analytical method were met. The method is acceptable.

Reference:	KCP 5.2/02
Report	Independent Laboratory Validation of Multi-Residue Method QuEChERS for

	Determination of Prothioconazole-desthio in Different Matrices of Plant Origin, N. Boubakri, 2023, Report No. S21-08354
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The method used was in line with the primary method (J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)). There were no deviations.

Results and discussions
The method for the determination of prothioconazole-desthio in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 17: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Dried beans	Prothioconazole-desthio	0.005	88	3	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Dried beans	Prothioconazole-desthio	0.05	89	4	

Table A 18: Characteristics for the analytical method used for independent laboratory validation of prothioconazole residues in crops

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio were less than 30% of the limit of quantification (> 30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points
Calibration range	0.150 – 6.0 ng/mL for dired beans equivalent to 0.0015 – 0.06 mg/kg
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOD: 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion
The method for the determination of prothioconazole-desthio in crops has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for the primary method (J. Pearson, 2022, Report No. QG/20/011, KCP 5.2/01 (filed in KCP 5.1.2/01)).

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

Comments of zRMS:	The method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole-desthio (as sum of isomers) in five (milk, egg, fat, liver and meat) different matrices of food of animal origin with the LOQ of 0.004 mg/kg for milk and 0.01 mg/kg for remaining matrices. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30%

	for all matrices. The method is acceptable.

Reference:	KCP 5.2/03
Report	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Food of Animal Origin, D. Kleinhenz, 2023, Report No. S21-08355
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Quantitative determination of prothioconazole-desthio in food of animal origin was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Samples were extracted with acetonitrile after addition of water with a ratio of 2 mL of extraction solvent per g of matrix. Liquid/liquid partition was performed by addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation. Clean-up was performed by purification of an aliquot of the acetonitrile extract by dispersive SPE with primary/secondary amine (PSA) and freezing out for fat matrix. The sample concentration in final extracts was 0.5 g sample per mL of extract. Quantification was performed by LC-MS/MS.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu			
Pre-column	HPLC guard column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)			
Column	Kinetex® 5µm XB-C18 100 Å (150 mm x 4.6 mm, 5 µm, Phenomenex, Art. No. 00F-4605-E0)			
Column oven temperature	40 °C			
Injection volume	10 µL			
Mobile phases	Eluent A: Water + 0.1 % formic acid (v/v) Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [mL/min]
	0.0	40	60	1.0
	1.0	40	60	1.0
	5.0	10	90	1.0
	6.0	10	90	1.0
	6.1	0	100	1.0
	7.0	0	100	1.0
	7.1	40	60	1.0
	9.0	40	60	1.0
Divert valve	To mass spectrometer from 4.5 min to 6.5 min			
Retention time	Prothioconazole-desthio: approx. 5.5 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	5500 V		Ionspray turbo heater (TEM)		650 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole-desthio	312 → 70#	126	10	47	12	100
	312 → 125	126	10	49	6	100

proposed for quantification. Both of the mass transitions listed can be used for quantification

Results and discussions

The analytical method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 312→70 and m/z 312→125). Due to enhanced sensitivity mass transition m/z 312→70 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.06 ng/mL to 6 ng/mL for milk and 0.15 ng/mL to 15 ng/mL for the remaining matrices. This range corresponds to a mass fraction level of 0.0012 mg/kg to 0.12 mg/kg for milk and 0.003 mg/kg to 0.3 mg/kg for the remaining matrices and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.999.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.004 mg/kg and 0.04 mg/kg for milk and 0.01 mg/kg to 0.1 mg/kg for the remaining matrices for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression or

enhancement was < ± 20 % for all matrices and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts
The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ
The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.004 mg/kg was confirmed for prothioconazole-desthio in milk and an LOQ of 0.01 mg/kg was confirmed for the remaining matrices. The LOD was defined as the lowest calibration standard, corresponding to 0.0012 mg/kg for milk and 0.003 mg/kg for the remaining matrices, which is 30 % of the LOQ.

Table A 19: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Comments
Milk	0.004	104	2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.04	97	2	
Egg	0.01	100	1	
	0.1	98	1	
Fat	0.01	85	4	
	0.1	83	3	
Liver	0.01	98	2	
	0.1	91	2	
Meat	0.01	101	2	
	0.1	97	2	

Table A 20: Characteristics for the analytical method used for validation of prothioconazole-desthio in food of animal origin

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 5 calibration points.
Calibration range	0.06 – 6 ng/ml for milk and 0.15 ng/ml – 15 ng/ml for the other matrices. This corresponds to 0.0012 – 0.12 mg/kg for milk and 0.003 – 0.3 mg/kg for the other matrices. r ≥ 0.999
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	LOQ = 0.004 mg/kg (milk) and 0.01 mg/kg (all other matrices) LOD = 0.0012 mg/kg (milk) and 0.003 mg/kg (all other matrices)

Conclusion
The validation criteria for the method used to determine prothioconazole-desthio in food of animal origin

has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The analytical method S21-08355 was successfully independently validated for the determination of residues of prothioconazole-desthio in milk and fat with LOQ of 0.004 mg/kg and 0.01 mg/kg, respectively. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% at each level. The acceptance criteria of the SANTE/2020/12830 rev.1 for the analytical method were met. The method is acceptable.

Reference:	KCP 5.2/04
Report	Independent Laboratory Validation of Prothioconazole-desthio in Different Matrices of Animal Origin, T. Rastogi, 2023, Report No. S21-08868
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The method used was in line with the primary method above. There were no deviations.

Results and discussions
The method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 21: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Milk	0.004	81.0	11	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.04	88.2	6.8	
Fat	0.01	78.5	4.6	
	0.10	75.4	4.7	

Table A 22: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in food of animal origin

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 8 calibration points.
Calibration range	0.060 - 6.0 ng/mL for milk and 0.150 - 15.0 ng/mL for fat matrix. Corresponding to 0.0012 - 0.12 mg/kg for milk and 0.003 - 0.3 mg/kg for fat. r ≥ 0.99
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.04 mg/kg (milk) and 0.01 mg/kg (fat) LOD = 0.0012 mg/kg (milk) and 0.003 mg/kg (fat)

Conclusion
The method for the determination of prothioconazole-desthio in food of animal origin has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for D. Kleinhenz, 2023 (Report No. S21-08355).

Comments of zRMS:	Two methods have been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole-desthio in and (5) hydroxy metabolites in honey with the LOQ of 0.005 mg/kg for each analyte. Mean recoveries were in the range of 60 – 120% with relative standard deviations of ≤30% for all analyte. The methods are acceptable.

Reference:	KCP 5.2/05
Report	Prothioconazole-desthio and hydroxy metabolites: Method Validation in Honey, J. Hitchens, 2023, Report No. QG/21/009
Guideline(s):	Yes - SANTE/2020/12830 rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole-desthio and hydroxy metabolites in honey was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Residues of prothioconazole-desthio were extracted from honey by dissolving the samples in water and then extracting the analyte with acetonitrile using QuEChERS kits. A portion of the final extract was then taken through the SPE clean-up and then diluted with water 2:3 v/v for final determination by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

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

<u>Example Instrument Conditions</u>

Liquid Chromatography:

Column:	Kinetex 5 µm XB-C18 100A, 150 x 4.6 mm		
Guard Column:	C18 4 x 3.0 mm – Part no. AJ0-4287		
Column Oven Temperature (°C):	40		
Injection Volume * (µL):	60		
Flow Rate (µL/min):	1000		
Mobile Phase A:	0.1 % Formic acid in water		
Mobile Phase B:	0.1 % Formic acid in methanol		
Gradient:	Time (minutes)	% Mobile Phase A	% Mobile Phase B
	0.00	40	60
	1.00	40	60
	5.00	10	90
	6.00	10	90
	6.10	0	100
	7.00	0	100
	7.10	40	60
	9.00	40	60
Flow divert:	Time (minutes)	Position	
	0.0	To waste	
	4.0	To mass spec	
	6.0 - End	To waste	
Autosampler Wash Solvent 1 (Strong):	0.1% Formic Acid in 2-Propanol/Acetonitrile/Water (1/1/1, v/v/v)		
Autosampler Wash Solvent 2 (Weak):	Methanol/Water (10/90, v/v)		

Mass Spectrometry:

MS/MS System	API 5000
Ionisation Mode:	ESI
Ion Source	Turbo Spray
Polarity	Positive
Scan Type	MRM
Ion Spray Voltage (IS) (V)	5500
Collision Gas (CAD)**	Medium
Source Temperature (TEM) (°C)	650
Curtain Gas (CUR)**	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50
Entrance Potential (EP) (V)	10
Declustering Potential (DP)** (V)	126

** Minor modifications are allowed to optimise instrument performance.

Analyte	Transition	Q1 Mass*	Q3 Mass*	Dwell Time (msec)	Collision Energy* (CE) (V)	Collision Exit Potential* (CXP) (V)	Declustering Potential (DP)	Expected Retention time (±0.5mins)
Prothioconazole -desthio	1#	312.1	70.1	250	20	10	126	5.3
	2#	312.1	125.0	250	30	15	126	

Transition 1 is to be used for sample quantification, transition 2 is for confirmation.

Results and discussions

The analytical method for the determination of prothioconazole-desthio and hydroxy metabolites in honey

has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity
Chromatographic interferences at the retention time of prothioconazole-desthio and (5) hydroxy metabolites were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples, demonstrating good selectivity of the method. In addition, LC-MS/MS monitoring two ion mass transitions is a highly specific technique. Product ion spectra for all analytes were obtained to justify the selection of ions used for analytical determinations.

Linearity
Calibration curves were obtained from a minimum of 8 calibration solutions containing prothioconazole-desthio and (5) hydroxy metabolites. For prothioconazole-desthio the standard calibration range was 0.05 to 2.5 ng/mL (equivalent to 0.00125 – 0.0625 mg/kg), covering a range from 25% of the LOQ to 25% above the highest concentration level.

For prothioconazole-desthio hydroxy metabolites the standard calibration range was 0.375 to 15.0 ng/mL (equivalent to 0.0015 – 0.060 mg/kg), covering a range from 30% of the LOQ to 20% above the highest concentration level.

Matrix matched standards were prepared with the exception of the extract stability analytical batch. Correlation coefficients $r \geq 0.995$ and coefficients of determination ($R2) \geq 0.99$ were achieved in the validation and extract stability batches using a linear regression with a 1/x weighting, demonstrating acceptable linearity.

Accuracy and Precision
Average recoveries and the overall average recovery were all within the acceptance range of 60-120% at the LOQ fortification level and 70-120% at the 10xLOQ fortification level, demonstrating satisfactory accuracy. The relative standard deviation (RSD) and the overall RSD did not exceed 30% at the LOQ fortification level and did not exceed 20% at the 10xLOQ fortification level, demonstrating satisfactory precision.

Matrix Effects
Matrix effects were investigated by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions. Experiments assessed whether or not matrix effects were significant (i.e. >20% enhancement or suppression). No significant matrix effects were observed. (i.e. >20% enhancement or suppression). However, matrix matched standards were used with the exception of the extract stability analytical batch.

Stability of Standards and Extracts
The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ
Prothioconazole-desthio: the limit of detection (LOD) was 0.00125 mg/kg (25% of LOQ) equivalent to the lowest calibration standard. Prothioconazole-desthio (5) hydroxy metabolites: the limit of detection (LOD) was 0.0015 mg/kg (30% of LOQ) equivalent to the lowest calibration standard. The limit of quantification (LOQ) was established at 0.005 mg/kg for prothioconazole-desthio and (5) hydroxy metabolites in honey, as confirmed by recovery efficiency testing.

Table A 23: Characteristics for the analytical method used for validation of prothioconazole-desthio and hydroxy metabolites in honey

	Prothioconazole-desthio	Prothioconazole-desthio hydroxy metabolites
Specificity	Chromatographic interferences at the retention time of prothioconazole-desthio and (5) hydroxy metabolites were either not detected (ND) or less than 30% of the limit of quantification (<30% LOQ) in reagent blank and duplicate control samples,	
Calibration (type, number of data points)	Minimum of 8 calibration points	
Calibration range	0.05 – 2.5 ng/ml (equivalent to 0.00125 – 0.0625 mg/kg) r ≥ 0.99	0.375 – 15.0 ng/ml (equivalent to 0.0015 – 0.060 mg/kg) r ≥ 0.99
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOD = 0.00125 mg/kg LOQ = 0.005 mg/kg	LOD = 0.0015 mg/kg LOQ = 0.005 mg/kg

Conclusion
The validation criteria for the method used to determine prothioconazole-desthio and hydroxy metabolites in honey has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The analytical methods of Hitchens were successfully independently validated for the determination of residues of prothioconazole-desthio in honey with LOQ of 0.005 mg/kg. The acceptance criteria of the SANTE/2020/12830 rev.1 for the analytical method were met. The method is acceptable.

Reference:	KCP 5.2/06
Report	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Honey, J. Wanger, 2023, Report No. S21-08357
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The method used was in line with the primary method. There were no deviations.

Results and discussions
The method for the determination of prothioconazole-desthio in honey has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 24: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Honey	0.005	105	2	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.05	110	2	

Table A 25: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in honey

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 9 calibration points.
Calibration range	0.050 - 3.0 ng/mL, corresponding to 0.00125 - 0.075 mg/kg $r \geq 0.999$
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.005 mg/kg LOD = 0.00125 mg/kg

Conclusion
The method for the determination of prothioconazole-desthio in honey has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for J. Hitchens, 2024, Report No. QG/21/008 and J. Hitchens, 2023, Report No. QG/21/009.

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

Comments of zRMS:	A analytical method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole and prothioconazole-desthio in soil with the LOQ of 0.006 mg/kg for prothioconazole and an LOQ of 0.002 mg/kg for prothioconazole-desthio. The method is acceptable.

Reference:	KCP 5.2/07
Report	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Soil, M. Kaiser, 2022, Report No. S21-08358
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole and prothioconazole-desthio in soil was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions
Liquid Chromatography:

Chromatographic conditions				
HPLC system	1290 Infinity II Binary LC System, Agilent Technologies			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenenx)			
Column	Agilent ZORBAX Eclipse XDB-C18 (50 x 4.6 mm, 1.8 µm, Part No. 927975-902)			
Column oven temperature	40 °C			
Injection volume	40 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	80	20	400
	0.3	80	20	400
	4.0	10	90	400
	6.5	10	90	400
	6.51	80	20	400
	8.5	80	20	400
Divert valve	to mass spectrometer from 5.5 min to 8.2 min			
Retention times	Prothioconazole: approx. 6.5 min Prothioconazole-desthio: approx. 6.2 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX API 6500+ System, AB SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V		Ionspray turbo heater (TEM)		450 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 189#	66	10	29	22	50
	344 → 154	66	10	43	16	50
Prothioconazole-desthio	312 → 70#	101	10	59	10	50
	312 → 125	101	10	45	14	50

proposed for quantification. Both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole and prothioconazole-desthio in soil has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 344→189 and m/z 344→154 for prothioconazole and m/z 312→70 and m/z 312→125 for prothioconazole-desthio). For prothioconazole mass transition m/z 344→189 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation and for prothioconazole-desthio m/z 312→70 accordingly.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to > 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched

calibration standards at a minimum of five (5) concentration levels ranging from 0.036 ng/mL to 36 ng/mL for prothioconazole and 0.12 ng/mL to 12 ng/mL for prothioconazole-dsesthio. This range corresponds to a mass fraction level of 0.0018 mg/kg to 0.18 mg/kg and 0.006 mg/kg to 0.6 mg/kg respectively and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.002, 0.006, 0.02 and 0.06 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression or enhancement was > ± 20 % for all matrices and thus deemed to be significant. Therefore, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ in soil of 0.006 mg/kg was confirmed for prothioconazole and an LOQ of 0.002 mg/kg for prothioconazole-desthio. The LOD was defined as the lowest calibration standard, corresponding to 0.002 mg/kg for prothioconazole and 0.0006 mg/kg for prothioconazole-desthio, which is 30 % of the LOQ.

Table A 26: Recovery results from method validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Soil	0.006	81	9	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.06	92	3	
Prothioconazole-desthio				
Soil	0.002	104	12	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.02	109	4	

Table A 27: Characteristics for the analytical method used for validation of prothioconazole and prothioconazole-desthio in soil

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were	

	either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.36 – 36 ng/mL, equivalent to 0.0018 - 0.18 mg/kg $r \geq 0.99$	0.12 - 12 ng/mL, equivalent to 0.006 - 0.6 mg/kg $r \geq 0.99$
Assessment of matrix effects is presented	Yes	
Limit of determination/quantification	LOQ = 0.006 mg/kg LOD = 0.002 mg/kg	LOQ = 0.002 mg/kg LOD = 0.0006 mg/kg

Conclusion
The validation criteria for the method used to determine prothioconazole and prothioconazole-desthio in soil has been met in accordance with SANTE/2020/12830 rev. 1.

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

Comments of zRMS:	A analytical method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole and prothioconazole-desthio in drinking water and surface water with the LOQ of 0.05 µg/L. The method is acceptable.

Reference:	KCP 5.2/08
Report	Development and Validation of an Analytical Method for Determination of Prothioconazole and Prothioconazole-desthio in Water, M. Kaiser, 2022, Report No. S21-08359
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole and prothioconazole-desthio in drinking water and surface water was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	a) HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu b) 1290 Infinity II HPLC, Agilent			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)			
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl (50 x 2.1 mm, 1.8 µm, Part No. 959757-912)			
Column oven temperature	40 °C			
Injection volume	40 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	60	40	400
	0.3	60	40	400
	1.3	20	80	400
	4	5	95	400
	6.5	5	95	400
	6.51	60	40	400
	8.5	60	40	400
Divert valve	a) to mass spectrometer from 1.7 min to 3.0 min b) to mass spectrometer from 1.0 min to 3.5 min			
Retention times	Prothioconazole: approx. 2.4 min Prothioconazole-desthio: approx. 2.2 min			

Mass Spectrometry:

Mass spectrometric conditions (both matrices)						
MS system	a) SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer) b) SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive Ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)		Ionspray turbo heater (TEM)		450 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole	344 → 154#	66	10	43	16	50
	344 → 189	66	10	29	22	50
Prothioconazole-desthio	312 → 70#	101	10	59	10	50
	312 → 125	101	10	45	14	50

used for quantification, but both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole and prothioconazole-desthio in surface and drinking water has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions per analyte (m/z 344→189 and m/z 344→154 for prothioconazole and m/z 312→70 and m/z 312→125 for prothioconazole-desthio). For prothioconazole mass transition m/z 344→154 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation and for prothioconazole-desthio m/z 312→70 accordingly.

A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to > 30 % of the LOQ at the retention time of the analyte in any of the five investigated matrices, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.0135 ng/mL to 1.35 ng/mL This range corresponds to a mass fraction level of 0.0149 µg/kg to 1.49 µg/kg mg/kg respectively and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). For each matrix, five (5) recovery determinations were performed at the LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.002, 0.006, 0.02 and 0.06 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev.1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Regarding prothioconazole matrix enhancement was at least partially ≥ + 20 % and deemed to be significant for both matrices. Therefore, matrix matched standards were used for quantification throughout the study. Regarding prothioconazole-desthio, matrix effects were < 20 % for both matrices and this deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study for both matrices.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.05 µg/L was confirmed for prothioconazole and prothioconazole-desthio in drinking and surface water.

The LOD was defined as the lowest calibration standard, corresponding to 0.0149 µg/L for all matrices, which is ≤ 30 % if the LOQ.

Table A 28: Recovery results from method validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level (~~mg/kg~~ µg/L) (*n* = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Drinking water	0.05	78	6	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	98	1	
Surface water	0.05	78	4	
	0.5	99	2	
Prothioconazole-desthio				
Drinking water	0.05	102	4	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	103	3	
Surface water	0.05	108	3	

	0.5	103	1	

Table A 29: Characteristics for the analytical method used for validation of prothioconazole and prothioconazole desthio residues in surface and drinking water

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.0135 – 1.35 ng/mL, equivalent to 0.0149 – 1.49 µg/L $r \geq 0.99$	
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOQ = 0.05 µg/L LOD = 0.0149 µg/L	

Conclusion
The validation criteria for the method used to determine prothioconazole and prothioconazole-desthio in surface water and drinking water has been met in accordance with SANTE/2020/12830 rev. 1.

Independent laboratory validation

Comments of zRMS:	The analytical method of M. Kaiser was successfully independently validated for the determination of residues of prothioconazole and prothioconazole-desthio in drinking water with LOQ of 0.05 µg/L. The acceptance criteria of the SANTE/2020/12830 rev.1 for the analytical method were met. The method is acceptable.

Reference:	KCP 5.2/09
Report	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole-desthio in Water, S. Jooß, 2023, Report No. S21-08869
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
The method used was in line with the primary method. There were no deviations.

Results and discussions
The method for the determination of prothioconazole and prothioconazole-desthio in water has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Table A 30: Recovery results from independent laboratory validation of prothioconazole and prothioconazole-desthio using the analytical method

Matrix	Fortification level (~~mg/kg~~ µg/L) (*n* = 5)	Mean recovery (%)	RSD (%)	Comments
Prothioconazole				
Drinking water	0.05	97.5	4.2	All mean recoveries were within

	0.5	98	1	the acceptable range of 70 – 120 % with RSD ≤ 20 %.
Prothioconazole-desthio				
Drinking water	0.05	100	3	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.5	100	2.5	

Table A 31: Characteristics for the analytical method used for independent laboratory validation of prothioconazole and prothioconazole-desthio residues in water

	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.
Calibration (type, number of data points)	Minimum of 6 calibration points.
Calibration range	0.0135 - 1.35 ng/mL, corresponding to 0.0149 – 2.49 µg/kg r ≥ 0.999
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ = 0.05 µg/L LOD = 0.0149 µg/L

Conclusion
The method for the determination of prothioconazole-desthio in water has been fully validated in accordance with SANTE/2020/12830 rev. 1 and can serve as an ILV for M. Kaiser, 2023, Report No. S21-08359

A 2.1.2.5 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)

Comments of zRMS:	The method has been successfully validated according to the guidance document SANTE/2020/12830 rev. 1 for the determination of prothioconazole-desthio in urine with the LOQ of 0.01 mg/L. Mean recoveries were in the range of 70 – 120% with relative standard deviations of ≤20%. The method is acceptable.

Reference:	KCP 5.2/10
Report	Development and Validation of an Analytical Method for Determination of Prothioconazole-desthio in Urine, N. Boubakri, 2023, Report No, S21-08361
Guideline(s):	Yes – SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods
Quantitative determination of prothioconazole-desthio in urine was determined by LC-MS/MS in accordance with SANTE/2020/12830 rev. 1.

Instrument Conditions

Liquid Chromatography:

Chromatographic conditions				
HPLC system	HPLC pump LC-30 AD, autosampler SIL-30ACMP, Shimadzu			
Pre-column	UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)			
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl (50 x 2.1 mm, 1.8 µm, Part No. 959757-912)			
Column oven temperature	40 °C			
Injection volume	5 µL			
Mobile phases	Eluent A: Water containing 10 mM Ammonium formate and 0.1 % formic acid Eluent B: Methanol			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	60	40	400
	0.3	60	40	400
	1.3	20	80	400
	4	5	95	400
	6.5	5	95	400
	6.51	60	40	400
	8.5	60	40	400
Divert valve	to mass spectrometer from 1.7 min to 3.5 min			
Retention times	Prothioconazole-desthio: approx. 2.5 min			

Mass Spectrometry:

Mass spectrometric conditions						
MS system	SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive Ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4500 V (pos)		Ionspray turbo heater (TEM)		450 °C	
Curtain gas (CUR)	Nitrogen set at 30 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 50 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
Prothioconazole-desthio	312 → 70#	101	10	59	10	50
	312 → 125	101	10	45	14	50

proposed for quantification. Both of the mass transitions listed can be used for quantification.

Results and discussions

The analytical method for the determination of prothioconazole-desthio in urine has been fully validated in accordance with SANTE/2020/12830 rev. 1.

Specificity

LC-MS/MS determination was conducted with evaluation by two (2) mass transitions (m/z 312→125 and m/z 312→70). Due to enhanced sensitivity mass transition m/z 312→70 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.

A reagent blank and two (2) control samples were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference above 30 % of LOQ at the retention time of prothioconazole-desthio in urine, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.3 ng/mL to 30 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/L to 0.3 mg/L and thus covers the range from no more than 30% of the limit of quantification (LOQ) and at least + 20% of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of

magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, correlation coefficients (R) were ≥ 0.99.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

All mean recovery values at fortification levels of 0.01 mg/L and 0.1 mg/L for two (2) mass transitions are within 70 % - 120 % with relative standard deviations ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards with solvent standards at the same nominal concentration. Matrix suppression was < 20% for prothioconazole-desthio in urine and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.

Stability of Standards and Extracts

The stability of stock solutions and stability of extracts was assessed and determined to be stable.

LOD and LOQ

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/L was confirmed for prothioconazole-desthio in urine. The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/L, which is 30% of the LOQ.

Table A 32: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification level (mg/kg) (*n* = 5)	Mean recovery (%)	RSD (%)	Comments
Urine	0.01	88	1	All mean recoveries were within the acceptable range of 70 – 120 % with RSD ≤ 20 %.
	0.1	92	2	

Table A 33: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in urine

	Prothioconazole	Prothioconazole-desthio
Specificity	Chromatographic interferences at the retention time of the relevant analytes were either not detected (ND) or less than 30% of the limit of quantification (>30% LOQ) in reagent blank and duplicate control samples.	
Calibration (type, number of data points)	Minimum of 5 calibration points.	
Calibration range	0.03 ng/mL – 30 ng/mL, equivalent to 0.003 mg/L – 0.3 mg/L r ≥ 0.99	
Assessment of matrix effects is presented	yes	
Limit of determination/quantification	LOQ = 0.01 mg/L LOD = 0.003 mg/L	

Conclusion
The validation criteria for the method used to determine prothioconazole-desthio in urine has been met in accordance with SANTE/2020/12830 rev. 1.

A 2.1.2.7 Other Studies/ Information

No new or additional studies have been submitted.