

# REGISTRATION REPORT

## **Part B**

### **Section 5**

#### **Analytical Methods**

Detailed summary of the risk assessment

Product code: CA3301

Product name(s): JOUST

Chemical active substance:

Prothioconazole, 250 g/L

Central zone

Zonal Rapporteur Member State: Poland

#### **CORE ASSESSMENT**

New Authorisation (Art.33)

Applicant: Nufarm Polska Sp. z o. o.

Submission date: 23/12/2021, updated September 2022

MS Finalisation date: October 2022 (initial Core Assessment)

January 2023 (final Core Assessment)

### Version history

When	What
December 2021	First submission
September 2022	Addition of information under 5.1.2/10
October 2022	Updated information under 5.2.1
October 2022	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 <del>struck through and shaded for transparency</del> .
January 2023	Final report (Core Assessment updated following the commenting period). Additional information/assessments included by the zRMS in the report in response to comments received from the cMS and the Applicant are <b>highlighted in yellow</b> . Information no longer relevant <del>is struck through and shaded</del> .

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## 5 Analytical methods

### 5.1 Conclusion and summary of assessment

#### **zRMS conclusions:**

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-prothioconazole-desthio 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, 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 <sup>3</sup> Additional method: Maasfeld 2002b (HPLC-MS/MS, JAU6476-desthio) LOQ Air: 0.0006 mg/m <sup>3</sup>
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) is not sufficient to monitor these lowered MRLs for food of plant origin. To cover the current residue definition and MRL limits, the Applicant should provide a suitable monitoring method including confirmation and ILV for all major matrix groups with a LOQ of 0.01 mg/kg for the determination of prothioconazole in plant commodities.

Regarding comment received from the CMS-DE Applicant has been requested by the zRMS for additional clarification.

**Applicant:**

*The product is intended to be used on high starch/dry grains and high oil commodities. The lowest MRL of these relevant groups is established as 0.04 mg/kg in gold of pleasure seeds. The LOQ of the EU agreed primary methods by Weeren (2000) is validated at 0.02 mg/kg with an ILV.*

*Consequently, the monitoring methods provided comply with the specific intended uses stated in the dRR.*

**zRMS:**

In our opinion, the EU agreed primary methods by Weeren (2000) with LOQ of 0.02 mg/kg with an ILV are sufficient for intended uses for Joust 250 EC.

It should be noted that with the study by Winter & Giesler (2017, S16-04434), the Applicant has provided a suitable monitoring method, including confirmation for all major matrix groups with a lower LOQ equals 0.01 mg/kg. However, an ILV of this method is missing. In our opinion, an ILV to this method should be provided by the Applicant as a post-registration requirement.

**Note:**

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.

The active substance prothioconazole was evaluated at the EU level according to the old data requirements. The Commission Regulation (EU) No 284/2013 is applicable now.

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.

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.

Additionally, an independent laboratory validation (ILV) for the method for the determination of residues of prothioconazole in drinking water is missing. Based on the indication of the SANTE/2020/12830, Rev.1 24. February 2021, the ILV for drinking water should be submitted.

In our opinion, it is necessary to supply the above-mentioned methods for determining the residues of prothioconazole in body fluids and tissues, in eggs, and ILV for drinking water at the renewal of the active substance and/or re-evaluation of plant production product.

Regarding comment on data gaps received from the cMS-DE Applicant has been requested by the zRMS for additional clarification.

**Applicant:**

*Nufarm position on the above mentioned data gaps is in accordance to the conclusion of the zRMS, and has been expressed accordingly during the commenting phase.*

*Nufarm, will provide the above-mentioned methods latest at the timelines as indicated by the zRMS.*

*Further to this, Nufarm is cooperating with Prothioconazole Main Notifier at EU level and has an agreement in place which allows the Regulatory Authorities in EU27 to make use of certain studies submitted by the Main Notifier at EU27 in the course of the renewal of prothioconazole or at Member State level, in support of this application. This is confirmed by the LoAs already included in the course of this submission.*

*All the data gaps identified by the zRMS will be fully covered by a new extended LoA to study summaries that will be available to Nufarm very early in 2023. These can be provided to any Member State, including the zRMS at that time in the course of this application for the registration of CA3301, at the time of renewal of Prothioconazole, or latest at the time of submission of the dossier in the course of the Art.43 for the re-registration of CA3301.*

**zRMS:**

zRMS maintains its position regarding the provision of missing data at the renewal of the active substance and/or re-evaluation of plant production product.

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:

- an analytical method for the determination of prothioconazole in body fluids and tissues is required according to the Commission Regulation (EU) No 284/2013 and should be provided at the renewal of the active substance and/or re-evaluation of plant production product;
- fully validated analytical method for the determination of prothioconazole-desthio in eggs is required according to the conclusions presented in EFSA Journal 2014;12(5):3689 and should be provided at the renewal of the active substance and/or re-evaluation of plant production product;
- an ILV of the method of determination of prothioconazole in drinking water is required according to the requirement of SANTE/2020/12830 and should be provided at the renewal of the active substance and/or re-evaluation of plant production product.
- an ILV of the method (Winter & Giesler (2017, S16-04434)) of determination of prothioconazole in all major matrix groups with an LOQ of 0.01 mg/kg is required according to the requirement of SANTE/2020/12830 and should be provided as a post-registration requirement.

Commodity/crop	Supported/ Not supported
Dry Commodity/Barley, Oat, Wheat, Triticale, Rye, Flax	Supported
High Oil Commodity/Oilseed Rape, Mustard, Cameline and other seed-producing Brassicaceae	Supported

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

### 5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of zRMS:	The method was successfully validated according to Guideline SANCO/3030/99 rev.5 and is acceptable for the quantification of prothioconazole in CA3301.
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Reference:	KCP 5.1.1/01
Report	Validation of Analytical Methodology for the Assay of Active Ingredient and Impurities in Prothioconazole 250 EC, NUL 3390 (CA3301), Ge, H., 2019, Report No. ABC-2019-020
Guideline(s):	EU SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes
Reference:	KCP 5.1.1/02
Report	Amendment No. 03 to Validation of Analytical Methodology for the Assay of Active Ingredient and Impurities in Prothioconazole 250 EC, NUL 3390 (CA3301), Wang Q., 2022, Report No. ABC-2019-020
Guideline(s):	EU SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

### Materials and methods

Samples of CA3301 test item (100 mg) are dissolved with 1 mL internal standard dimethylphthalate at 0.03 g/mL in acetonitrile and made up to volume with acetonitrile (50 mL). The samples are analysed for prothioconazole content by HPLC-UV, using a Eclipse Plus C8 column (100 mm x 4.6 mm, 3.5 µm). Calibration standards are prepared by dissolving amounts of the prothioconazole reference item (25 mg) with 1 mL internal standard dimethylphthalate at 0.03 g/mL in acetonitrile and made up to volume with acetonitrile (50 mL).

### Analytical conditions

System: Agilent 1260 HPLC with DAD  
Column: Eclipse Plus C8 column (100 mm x 4.6 mm, 3.5 µm)  
Mobile phase A: water (0.1% H<sub>3</sub>PO<sub>4</sub>)  
Mobile phase B: Acetonitrile  
Isocratic: A/B 45/55 v/v at 0.5 mL/min  
Column temperature: 35°C  
Injection volume: 3 µL  
Detection: UV at 254 nm

Quantitation was performed by the following equation:

$$W'' = \frac{R}{R'} \times W' \times P$$

$$\text{Active Ingredient (\%)} = \frac{W''}{W} \times 100$$

Where:

R = the average peak area ratio of Active Ingredient to ISTD in Test Item solution;

R' = the average peak area ratio of Active Ingredient to ISTD in Prothioconazole standard solution;

W = the mass of Test Item;

W' = the mass of Prothioconazole standard;

P = the purity of Prothioconazole standard

## Validation - Results and discussions

**Table 5.2-1: Methods suitable for the determination of active substance prothioconazole in plant protection product CA3301**

	Prothioconazole			
<b>Author(s), year</b>	Ge, H. (2019)			
<b>Principle of method</b>	HPLC-UV			
<b>Linearity</b> (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of detector response was demonstrated using injections of five concentrations (in duplicate) of reference standard in the approximate range of 395 to 637 mg/L (equivalent to 19.8% w/w – 31.8% w/w prothioconazole in the test item), with a correlation coefficient (r) of 0.9997 (slope = 0.0091, intercept = 0.0224).			
		mg pure prothioconazole in L acetonitrile	% w/w pure prothioconazole in product CA3301*	g prothioconazole in L product CA 3301
	Lower standard	395 mg/L	19.8% w/w	196.5 g/L
	Higher standard	637 mg/L	31.8% w/w	316.7 g/L
	Nominal*	503 mg/L	25.1% w/w	250 g/L
	± 20% of nominal conc.	402-604 mg/L	20.1-30.2%	200 – 300 g/L
* Considering a test item solution at 2 mg product / L acetonitrile (100 mg in 50 mL)				
** Considering a density of 0.9948 for product CA3301				
<b>Precision – Repeatability Mean n = 5 (%RSD)</b>	Repeatability (precision) was determined from single determinations of five samples of CA3301. Mean content: 25.45% RSD: 0.39% RSDr: 1.65% Hr (Horrat value): 0.24			
<b>Accuracy n = 5 at 3 levels (% Recovery)</b>	Not evaluated as part of this study:			
	<b>Fortification Level</b>			<b>n</b>
	mg pure prothioconazole in L acetonitrile	% w/w pure prothioconazole in product CA3301*	g prothioconazole in L product CA 3301	<b>Mean Recovery (%)</b>
	450	22.5	225	5
	500	25	250	5
	550	27.5	275	5
<b>RSD (%)</b>				
100.08				
99.68				
100.10				
<b>Interference/ Specificity</b>				
Samples of blank formulation, reference item solution and test item solution were				

	Prothioconazole
	analysed. No additional analytical signals in the mean retention time of the active substance were observed. Relevant chromatograms are provided.
Comment	-

## Conclusion

The validation of the method for analysis of prothioconazole in CA3301 has not been previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3030/99 rev.5 and was successfully validated.

The method is acceptable for the quantification of prothioconazole in CA3301.

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

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

Comments of zRMS:	The method was successfully validated according to Guideline SANCO/3030/99 rev.5 and is acceptable for the quantification of relevant impurities prothioconazole-desthio and toluene in CA3301.
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Reference:	KCP 5.1.1/01
Report	Validation of Analytical Methodology for the Assay of Active Ingredient and Impurities in Prothioconazole 250 EC, NUL 3390 (CA3301), Ge, H., 2019, Report No. ABC-2019-020
Guideline(s):	EU SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary

## Materials and methods

Samples of CA3301 test item (100 mg) are dissolved in acetonitrile (10 mL). The samples are analysed for prothioconazole-desthio content by HPLC-MS, using a Eclipse Plus C8 column (100 mm x 4.6 mm, 3.5 µm). The samples are analysed for toluene content by GC-FID, using a DB-624 column (30 m x 0.32 mm, 1.8 µm). Calibration standards containing the respective impurity reference item are prepared in acetonitrile.

## Analytical conditions

### HPLC/MS

System: Agilent 6130 HPLC/MS with EI  
Column: Eclipse Plus C8 (100 mm x 4.6 mm, 3.5 µm)  
Mobile phase A: water (0.5% formic acid)  
Mobile phase B: Acetonitrile

Column temperature: 30°C

Injection volume: 2 µL

Detection: UV at 254 nm

MS source: API-ES

Polarity: positive

SIM ion: 312 – 314 at 14 min

	A	B
0 min	58	42
20 min	58	42
20.1 min	0	100
23 min	0	100
23.1 min	58	42

#### GC-FID

System: Agilent 7890 Gc with FID

Column: DB-624 (30 m x 0.32 mm, 1.8 µm)

Carrier gas: nitrogen 2 mL/min

Column temperature: 70°C for 3 minutes then to

Quantitation was performed by the following equation:

$$W'' = \frac{A}{A'} \times W' \times P \times 0.001$$

Where:

A = the average peak area of impurity in sample solution;

A' = the average peak area of impurity in standard solution;

W = the mass of sample;

W' = the mass of impurity standard;

P = the purity of reference standard

300°C at 30°C/min held for 10 min

Injector temperature: 240°C

Injection volume: 1 µL (split 3:1)

Detection: FID at 300°C

$$\text{Impurity (\%)} = \frac{W''}{W} \times 100$$

### Validation - Results and discussions

**Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) CA3301**

	Prothioconazole-desthio	Toluene
<b>Author(s), year</b>	Ge, H. (2019)	Ge, H. (2019)
<b>Principle of method</b>	HPLC-MS	GC-FID
<b>Linearity (linear between mg/L) (correlation coefficient, expressed as r)</b>	The linearity of detector response was demonstrated using injections of five concentrations (in duplicate) of reference standard in the approximate range of 0.306 to 55.0 mg/L (equivalent to 0.00306% w/w – 0.550% w/w prothioconazole-desthio in the test item), with a correlation coefficient (r) of 0.9996 (slope = 1286052, intercept = 562946).	The linearity of detector response was demonstrated using injections of six concentrations (in duplicate) of reference standard in the approximate range of 1.90 to 309 mg/L (equivalent to 0.0190% w/w – 3.09% w/w toluene in the test item), with a correlation coefficient (r) of 1.0000 (slope = 5.5256, intercept = 0.0838).
<b>Precision – Repeatability Mean n = 5 (%RSD)</b>	Repeatability (precision) was determined from single determinations of five samples of CA3301. Mean content: 0.0058% w/w RSD: 5.17% RSDr: 5.82% Hr (Horrat value): 0.89	Repeatability (precision) was determined from single determinations of five samples of CA3301. Mean content: 0.026% w/w RSD: 3.85% RSDr: 4.64% Hr (Horrat value): 0.83
<b>Accuracy n = 5 (% Recovery)</b>	Recovery was determined using replicate injections of test item which had been fortified with known concentrations of prothioconazole-desthio at two fortification levels. The results are presented in Table 5.2-3 below.	Recovery was determined using replicate injections of test item which had been fortified with known concentrations of toluene at two fortification levels. The results are presented in Table 5.2-4 below.
<b>Interference/ Specificity</b>	Samples of blank formulation, reference item solution and test item solution were analysed. No additional analytical signals in the mean retention time of the active substance were observed. Relevant chromatograms are provided.	Samples of blank formulation, reference item solution and test item solution were analysed. No additional analytical signals in the mean retention time of the active substance were observed. Relevant chromatograms are provided.
<b>LOQ</b>	The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable accuracy and precision data is obtained, was determined to be 0.004367% w/w.	The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable accuracy and precision data is obtained, was determined to be 0.019008% w/w.
<b>LOD</b>	The limit of detection was calculated to be 0.000023% w/w (three times signal to noise ratio).	The limit of detection was calculated to be 0.0041% w/w (three times signal to noise ratio).
<b>Comment</b>	-	-

**Table 5.2-3: Prothioconazole-desthio Accuracy data - Ge, H. (2019)**

Fortification Level (mg/L)	No of Determinations	Mean Recovery (%)	RSD (%)	Acceptable Recovery (%)
0.4367 (0.004367% w/w)	5	120.90	1.58	75 – 125*
3.2457 (0.032457% w/w)	5	112.83	0.62	75 – 125

\* 70-130% according to SANCO 3030/99 as % w/w in test item is below 0.01%.

**Table 5.2-4: Toluene Accuracy data - Ge, H. (2019)**

Fortification Level (mg/L)	No of Determinations	Mean Recovery (%)	RSD (%)	Acceptable Recovery (%)
1.9008 (0.019008% w/w)	5	88.50	4.68	75 – 125
205.9872 (2.05% w/w)	5	108.25	1.16	90 – 110

## Conclusion

The validation of the method for analysis of relevant impurities prothioconazole-desthio and toluene in CA3301 has not been previously evaluated at EU level. It was performed under GLP according to Guideline SANCO/3030/99 rev.5 and was successfully validated.

The method is acceptable for the quantification of relevant impurities prothioconazole-desthio and toluene in CA3301.

Refer to part C for analytical methods for non relevant impurities and study report..

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

CA3301 does not contain any (eco)toxicologically relevant formulants.

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

Prothioconazole [745] Handbook P:

Outline of method

The content of prothioconazole (g/kg) is determined by reversed phase high performance liquid chromatography using UV detection at 254 nm and external standard calibration.

Scope

The method is usable for TC, EC, FS and SC-formulations

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

An overview on the acceptable methods and possible data gaps for analysis of residues of prothioconazole for the generation of pre-authorization data is given in the following table. For the detailed evaluation of 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-desthio (sum of isomers)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products,... (Residues)	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.1.2/01 Winter O., 2017, report No. S16-04434 (NUD-1601V)
	Confirmatory	prothioconazole and prothioconazole- desthio (wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber)	Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	

Component of residue definition: Prothioconazole-desthio (sum of isomers)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.1.2/02 Winter O. & Nachtigall S, 2020, report No. S16-04435 (NUD-1602V)
	Confirmatory	Prothioconazole- $\alpha$ -hydroxy-desthio, prothioconazole 3-, -4-, -5- and -6-hydroxy-desthio in wheat (whole plant, grain and straw) and oilseed rape (seeds)	Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Primary	0.01 mg/kg	LC-DMS/MS/MS	KCP 5.1.2/03 Schernikau N., 2016, report No. S15-03542 (GAB-1537V)
	Confirmatory	1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid wheat (grain and straw), barley (grain and straw) grape (bunches) and oilseed rape	Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Primary	0.01 mg/kg	LC-DMS/MS/MS	KCP 5.1.2/04 Class, T., 2011, report No. P 2383G, M-420638-01-1
	Confirmatory	1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid wheat (grain and straw), barley (grain and straw) grape (bunches) and oilseed rape	Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Please refer also to post authorisation methods.			
Animal products, food of animal origin,... (Residues)	Please refer to post authorisation methods.			
Soil, water, sediment,... (Environmental fate)	Please refer to post authorisation methods.			
Soil, water,... (Efficacy)	Not applicable.			
Feed, body fluids,... (Toxicology) Body fluids, air,... (Exposure)	Prothioconazole is not classified as toxic or highly toxic and therefore analytical methods for the determination of residues in human and animal tissues and fluids are not required. This was confirmed in EFSA Scientific Report (2007) 106, 1-98, Conclusion on the peer review of prothioconazole.			
Soil, water,... (Ecotoxicology)	Primary	10 mg/kg	UHPLC-TOF-MS/MS	KCP 5.1.2/05 Morsiani S., 2020, report No. 20070-01R, analytical method: MA RES 147-1
	Confirmatory	prothioconazole (water)	Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Primary	0.018 mg/kg	UHPLC-TOF-MS/MS	KCP 5.1.2/06 Morsiani S., 2020, report No. 20070-02R, analytical method: MA RES 148-1
	Confirmatory	prothioconazole (sucrose solution)	Self confirmatory MS/MS. Not required for methods for risk assessment according to	

Component of residue definition: Prothioconazole-desthio (sum of isomers)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
			SANTE/2020/12830, Rev.1	
	Primary	0.257 µg/L prothioconazole (aqueous test medium)	HPLC-MS/MS	KCP 5.1.2/07 Semal S. (Study Director Dupont A.), 2021a, report No. 20190456
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Primary	0.389 µg/L prothioconazole (aqueous test medium)	HPLC-MS/MS	KCP 5.1.2/08 Semal S. (Study Director Dupont A.), 2021b, report No. 20190454
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
	Primary	0.7 mg prothioconazole/L (water)	HPLC-MS/MS	KCP 5.1.2/09 Ortiz M. G., 2021, report No. S20-09403
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev.1	
Water, buffer solutions,... (Properties)	Primary	0.5 mg prothioconazole/L (acetonitrile rinse and tank mix solutions)	HPLC-UV	KCP 5.1.2/10 Calvert A., 2022, report No. 22/1499
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev.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 in the plant protection product already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

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

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

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

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Cereals, green material/whole plant Rapeseed, whole plant (high water content)	Prothioconazole-desthio (sum of isomers)	LOQ: 0.01 mg/kg MRL not applicable	See studies presented B.7.2.3
Cereals, grains (high starch content/dry commodities)		Barley MRL: 0.2 mg/kg Oat MRL: 0.05 mg/kg Rye MRL: 0.05 mg/kg Wheat MRL: 0.1 mg/kg	Reg. (EU) 2019/552
Oilseed, seeds (high oil content)		Linseed MRL: 0.09 mg/kg Poppy MRL: 0.09 mg/kg Mustard MRL: 0.09 mg/kg Rapeseed MRL: 0.15 mg/kg Gold of pleasure MRL: 0.04 mg/kg	Reg. (EU) 2019/552
Cereal straw (dry commodities)		LOQ: 0.01 mg/kg MRL not applicable	See studies presented B.7.2.3
Ruminants, Muscle	Prothioconazole-desthio (sum of isomers)	0.01 mg/kg	Reg. (EU) 2019/552
Ruminants, Fat		0.02 mg/kg	
Ruminants, Liver, kidney		0.5 mg/kg	
Milk		0.01* mg/kg	
Poultry, Muscle		0.01* mg/kg	
Poultry, Fat		0.01* mg/kg	
Poultry, Liver, kidney		0.1 mg/kg	
Eggs		0.01* mg/kg	
Soil (Ecotoxicology)	Prothioconazole-desthio	0.212 mg/kg	NOEC value for Eisenia Foetida (EFSA Journal (2007) 106, 1-98)
Drinking water (Human toxicology)	Prothioconazole-desthio (sum of isomers)	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Prothioconazole and prothioconazole-desthio	0.308 mg/L prothioconazole 3.34 µg/L prothioconazole-desthio	NOEC value for O. mykiss (EFSA Journal (2007) 106, 1-98)
Air	Prothioconazole and prothioconazole-desthio	0.015 mg/m <sup>3</sup> prothioconazole 0.0006 mg/m <sup>3</sup> prothioconazole-desthio	(EFSA Journal (2007) 106, 1-98)

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Tissue (meat or liver)	N/A	not required	Not classified as T / T+
Body fluids		not required	Not classified as T / T+

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in plant matrices is given in the following tables. For the detailed evaluation of 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 (sum of isomers)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.02 mg/kg (tomato)	GC-MS	Weeren, P., 2000 / EU agreed
	ILV	0.02 mg/kg (tomato)	GC-MS	Class, 2001 / EU agreed
High acid content	Primary	0.02 mg/kg (orange)	GC-MS	Weeren, P., 2000 / EU agreed
	ILV	According to SANTE/2020/12830, if the primary method is identical for all matrix groups, it is sufficient to perform the ILV for commodities of two of these groups, one of them with high water content. This is applicable here.		
High oil content	Primary	0.02 mg/kg (rape seed)	GC-MS	Weeren, P., 2000 / EU agreed
	ILV	According to SANTE/2020/12830, if the primary method is identical for all matrix groups, it is sufficient to perform the ILV for commodities of two of these groups, one of them with high water content. This is applicable here.		
High protein/high starch content (dry)	Primary	0.02 mg/kg (wheat grain) and 0.05 mg/kg (wheat straw)	GC-MS	Weeren, P., 2000 / EU agreed
	ILV	0.02 mg/kg (wheat grain)	GC-MS	Class, 2001 / EU agreed
Difficult (if required, depends on intended use)	Not required.			

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
Not required, because:	According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required. Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### 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 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 (sum of isomers)				
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	xxxxxxxxxx, 2001c / EU agreed
	ILV	0.004 mg/kg	HPLC-MS/MS	xxxxxxxxxx, 2001 / EU agreed
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	xxxxxxxxxx 2001b / EU agreed
	ILV	0.01 mg/kg	HPLC-MS/MS	xxxxxxxxxx 2001 / EU agreed
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	xxxxxxxxxx 2001b / EU agreed
	ILV	0.01 mg/kg	HPLC-MS/MS	xxxxxxxxxx 2001 / EU agreed
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	KCP 5.2/01 Kalathoor, R., 2021, S20-09747, New study
	ILV		HPLC-MS/MS	KCP 5.2/02 Greiner, M., 2021, S21-02654, New study
	Confirmatory		Self confirmatory (MS/MS)	-

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
Not required, because:	Please refer to justification in Table 5.3-6.

### 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 studies it is referred to Appendix 2.

**Table 5.3-7: 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 (Prothioconazole, prothioconazole-desthio and prothioconazole-3-OH-desthio)	HPLC-MS/MS	Schramel, 2000 / EU agreed
Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	
Primary	0.01 mg/kg (Prothioconazole-desthio)	GC-MS	Steinhauser, 2001 / EU agreed
Confirmatory		-	

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

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in surface and drinking water is given in the following tables. For the detailed valuation of studies it is referred to Appendix 2.

**Table 5.3-8: 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)	HPLC-MS/MS	Sommer, 2001b / EU agreed
	Confirmatory	0.05 µg/L (prothioconazole-desthio)	Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	
Surface water	Primary	0.1 µg/L (prothioconazole)	HPLC-MS/MS	Sommer, 2001b / EU agreed
	Confirmatory	0.05 µg/L (prothioconazole-desthio)	Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	

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

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in air is given in the following tables. For the detailed evaluation of studies please refer to Appendix 2.

**Table 5.3-9: 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)	HPLC-MS/MS	Maasfeld, 2002a-2002b / EU agreed
Confirmatory	0.0006 µg/L (prothioconazole-desthio)	Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	

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

#### **5.3.2.6 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 studies it is referred to Appendix 2.

Prothioconazole is not classified as toxic or highly toxic and therefore analytical methods for the determination of residues in human and animal tissues and fluids are not required. This was confirmed in EFSA Scientific Report (2007) 106, 1-98, Conclusion on the peer review of prothioconazole.

#### **5.3.2.7 Other studies/ information**

No other studies submitted.

## Appendix 1 Lists of data considered in support of the evaluation

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2/01	Winter O., Giesler W.,	2017	Validation of an Analytical Method for the Determination of Prothioconazole (PTZ) and its Metabolite PTZ-desthio in Different Matrices of Plant Origin Report No. S16-04434 (NUD-1601V) Eurofins Agrosience Services Chem GmbH GLP Unpublished	N	Nufarm
KCP 5.1.2/02	Winter O., Nachtigall S	2020	Validation of an Analytical Method for the Determination of relevant Metabolites of Prothioconazole in Different Matrices of Plant Origin Report No. S16-04435 (NUD-1602V) Eurofins Agrosience Services Chem GmbH GLP Unpublished	N	Nufarm
KCP 5.1.2/03	Schernikau N.	2016	Validation of an Analytical Method for the Determination of Triazole and Triazole-based Metabolites in the Agricultural Commodity Wheat, Barley, Grape and Rape Report No. S15-03542 (GAB-1537V) Eurofins Agrosience Services Chem GmbH GLP Unpublished	N	Nufarm
KCP 5.1.2/04	Class, T.	2011	Modification M004 of BCS residue analytical method 01062 for the determination of 1,2,4-Triazole, Triazolylalanine, Triazole acetic acid and Triazole lactic acid by LC/DMS/MS/MS in plant materials Method 01062/M004, Report No. P 2383G, M-420638-01-1 PTRL Europe GmbH GLP Unpublished	N	TDMG
KCP 5.1.2/05	Morsiani S	2020	Analytical Method for the Determination of the content of Prothioconazole in water by UHPLC-TOF-MS/MS Test Site Code: 20070-01R (Study Code 029SRFR20C05), Analytical Method: MA RES 147-1 Renolab s.r.l. GLP Unpublished	N	Nufarm
KCP 5.1.2/06	Morsiani S	2020	Analytical Method for the Determination of the content of Prothioconazole in sucrose feeding solutions of honey bees by UHPLC-TOF-MS/MS	N	Nufarm

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
			Test Site Code: 20070-02R (Study Code 029SRFR20C04), Analytical Method: MA RES 148-1 Renolab s.r.l. GLP Unpublished		
KCP 5.1.2/07	Semal S.	2021	250 EC Prothioconazole (CA3301) – Effect on the Aquatic Higher Plant Lemna gibba in a 7-Day Growth Inhibition Test Report No. 20190456 Innovative Environmental Services (IES) Ltd GLP Unpublished	N	Nufarm
KCP 5.1.2/08	Semal S.	2021	250 EC Prothioconazole (CA3301) - Effect on Skeletonema sp. in a 72-Hour Algal Growth Inhibition Test Report No. 20190454 Innovative Environmental Services (IES) Ltd GLP Unpublished	N	Nufarm
KCP 5.1.2/09	Ortiz M. G.	2021	CA3301 (Prothioconazole 250 EC, NUL 3390): Honey Bee (Apis mellifera L.) Larval Toxicity Test following Repeated Exposure under Laboratory Conditions Report No. S20-09403 Eurofins Agrosience Services EcoChem GmbH GLP Unpublished	N	Nufarm
KCP 5.1.2/10	Calvert A.	2022	CA3301 – Effectiveness of Cleaning Report No. 22/1499 Nufarm UK Limited GLP Unpublished	N	Nufarm
KCP 5.2/01	Kalathoor, R.	2021	Amendment 1 to Final Report and Final Report Development and Validation of Analytical Methods for the Determination of Prothioconazole in different Matrices Report No. S20-09747 Eurofins Agrosience Services Chem GmbH GLP Unpublished	N	Nufarm
KCP 5.2/02	Greiner M.	2021	Independent Laboratory Validation of Analytical Methods for the Determination of Prothioconazole Metabolites in Honey Report No. S21-02654	N	Nufarm

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
			Eurofins Agrosience Services Chem GmbH GLP Unpublished		

**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
CP 5.1.2 CP 5.2	Weeren P.	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 Report No. 00086/M033 Dr. Specht & Partner GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	Class T.	2001	Independent laboratory validation of DFG method S19 (extended version) for the determination of residues of JAU 6476-dethio (BAYER method 00086/M033) in plant materials Report No. P/B 484 G PTRL Europe GmbH GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	xxxxxxxxxxxxxx	2001b	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio and JAU6476-desthio in/on matrices of animal origin by HPLC-MS/MS Report No. 00655, Date 2001-02-27 xxxxxxxxxxxxxx GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	xxxxxxxxxxxxxx	2001c	Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio and JAU6476-desthio in milk by HPLC-MS/MS Report No. 00655/M001 xxxxxxxxxxxxxx GLP Unpublished	N	BAY

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
CP 5.1.2 CP 5.2	xxxxxxxxxxxxxx	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 matreces of animal origin by HPLC-MS/MS Report No. A-14-01-01 xxxxxxxxxxxxxxxxxxxxxxxxxxxx GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	Schramel, O.	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 Report No. 00610 Bayer AG GLP	N	BAY
CP 5.1.2 CP 5.2	Steinhauser S.	2001	Enforcement method 00086/M038 for the determination of the residues of JAU 6476-desthio in soil – validation of DFG method S 19 (extended revision) Report No. 00086/M038 Dr. Specht & Partner GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	Sommer H.	2001b	Enforcement methd 00684 for determination of JAU 6476 and JAU 6476-desthio in drinking and surface water by HPLC-MS/MS Report No. 00684 Bayer AG GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	Maasfeld W.	2002	Method for the determination of JAU 6476 in air by HPLC-MS/MS Report No. 00724 Bayer AG GLP Unpublished	N	BAY

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

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

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

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

## Appendix 2 Detailed evaluation of submitted analytical methods

### A 2.1 Analytical methods for prothioconazole

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

##### A 2.1.1.1 Analytical method – Determination of prothioconazole in plant matrices

##### A 2.1.1.1.1 Method validation

Comments of zRMS:	<p>The method has been successfully validated according to the guidance documents SANCO/825/00, rev. 8.1, SANCO/3029/99 rev. 4 of the European Commission for the determination of prothioconazole and prothioconazole-desthio in wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber with the LOQ of 0.01 mg/kg calculated as prothioconazole-desthio.</p> <p>Mean recoveries were in the range of 70 – 110% with relative standard deviations of <math>\leq 20\%</math> for all analytes and matrices at each level.</p> <p>The study is acceptable.</p>
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Reference:	KCP 5.1.2/01
Report	Validation of an Analytical Method for the Determination of Prothioconazole (PTZ) and its Metabolite PTZ-desthio in Different Matrices of Plant Origin Winter O., Giesler W., 2017, report number S16-04434 (NUD-1601V)
Guideline(s):	SANCO/825/00 rev. 8.1 SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability:	Yes

### Materials and methods

Samples of wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber (5 g) were extracted with acetonitrile (10 mL) and if necessary, after addition of water. Ascorbic acid was added to cucumber matrix before extraction in order to stabilise prothioconazole during the extraction step. To the other matrices, ascorbic acid solution was added. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation an aliquot of the acetonitrile phase was diluted with methanol/water (2/3, v/v). A clean-up step was not necessary. Samples were analysed by LC-MS/MS (two mass transitions monitored per analyte; prothioconazole: 342/100 and 342/125 m/z, prothioconazole-desthio: 312/70 and 312/125 m/z). Matrix-matched standards were used for quantitation. The intended limit of quantification (LOQ) was 0.01 mg/kg (calculated as prothioconazole-desthio) in all matrix types.

### Analytical conditions

System: 1260 Infinity Binary LC System,  
Agilent Technologies  
Column: Luna C18(2) 100Å (150 mm x 2 mm, 5  
µm, Phenomenex  
Mobile phase A: Methanol  
Mobile phase B: Water + 10 mM ammonium  
acetate  
Flow: 0.6 L/min  
Column temperature: 50°C  
Injection volume: 20 µL

	A	B
0 min	50	50
4 min	95	5
6 min	95	5
6.1 min	50	50
8 min	50	50

System: SCIEX TripleQuad 5500 System, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type: Electrospray ionisation (ESI, TurboIon Spray)

Polarity: Positive/negative ion switching mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage (IS) 4500 V (pos) and - 4500 V (neg)

Prothioconazole: 342 → 100# (neg); 342 → 125 (neg)

Prothioconazole-desthio: 312 → 70# (pos); 312 → 125 (pos)

## Results and discussions

**Table A 1: Recovery results from method validation of prothioconazole using the analytical method**

Prothioconazole							
Matrix	Fortification Level	Recovery	Mean Recovery	Rel. Std. Dev.	Replicates	Overall Mean Recovery	Overall Rel. Std. Dev.
	(mg/kg)	(%)	(%)	(%)		(%)	(%)
Transition <i>m/z</i> 342→100 (Proposed for Quantification)							
Wheat (grain)	0.01	89, 87, 92, 87, 90	89	2.4	5	92	4.0
	0.1	96, 92, 95, 91, 98	94	3.1	5		
Grapes	0.01	88, 83, 78, 88, 87	85	5.1	5	89	6.5
	0.1	95, 96, 88, 96, 87	92	4.9	5		
Oilseed rape (seeds)	0.01	81, 75, 81, 81, 83	80	3.8	5	80	3.2
	0.1	83, 80, 78, 77, 80	80	2.9	5		
Bean (dry)	0.01	89, 89, 98, 96, 94	95	1.8	5	94	3.3
	0.1	94, 95, 94, 98, 94	88	4.5	5		
Cucumber	0.01	88, 89, 82, 93, 88	88	4.5	5	91	5.1
	0.1	92, 97, 95, 97, 91	94	3.0	5		
Transition <i>m/z</i> 342→125 (Proposed for Confirmation)							
Wheat (grains)	0.01	95, 73, 83, 83, 83	83	9.3	5	89	9.4
	0.1	98, 92, 94, 93, 99	95	3.3	5		
Grapes	0.01	85, 82, 72, 88, 86	83	7.6	5	87	8.3
	0.1	92, 94, 85, 97, 93	92	4.8	5		
Oilseed rape (seeds)	0.01	78, 78, 78, 81, 80	79	1.8	5	77	4.0
	0.1	77, 77, 75, 70, 79	76	4.5	5		
Bean (dry)	0.01	91, 85, 88, 92, 96	90	4.6	5	93	4.4
	0.1	96, 97, 95, 92, 97	95	2.2	5		
Cucumber	0.01	85, 89, 82, 96, 87	88	6.0	5	91	6.0
	0.1	91, 96, 98, 97, 90	94	3.9	5		

Prothioconazole-desthio							
Matrix	Fortification Level	Recovery	Mean Recovery	Rel. Std. Dev.	Replicates	Overall Mean Recovery	Overall Rel. Std. Dev.
	(mg/kg)	(%)	(%)	(%)		(%)	(%)
Transition <i>m/z</i> 312→70 (Proposed for Quantification)							
Wheat (grain)	0.01	94, 94, 96, 92, 104	96	4.9	5	96	3.5
	0.1	97, 93, 97, 97, 95	96	1.9	5		
Grapes	0.01	99, 107, 107, 110, 109	106	4.1	5	107	3.8
	0.1	103, 110, 110, 110, 102	107	3.9	5		
Oilseed rape (seeds)	0.01	73, 72, 76, 73, 73	73	2.1	5	75	4.4
	0.1	79, 78, 73, 81, 72	77	5.1	5		
Bean (dry)	0.01	98, 95, 95, 91, 97	95	2.8	5	98	4.2
	0.1	105, 99, 102, 100, 94	100	4.1	5		
Cucumber	0.01	94, 96, 90, 94, 92	93	2.4	5	95	3.1
	0.1	97, 97, 99, 98, 92	97	2.8	5		
Transition <i>m/z</i> 312→125 (Proposed for Confirmation)							
Wheat (grains)	0.01	89, 93, 90, 93, 88	91	2.5	5	94	3.8
	0.1	95, 96, 97, 97, 98	97	1.2	5		
Grapes	0.01	97, 94, 109, 102, 107	102	6.3	5	102	4.4
	0.1	99, 102, 100, 104, 102	101	1.9	5		
Oilseed rape (seeds)	0.01	79, 72, 71, 78, 76	75	4.7	5	77	4.9
	0.1	80, 78, 77, 83, 73	78	4.7	5		
Bean (dry)	0.01	103, 92, 92, 98, 97	96	4.8	5	98	4.1
	0.1	104, 98, 99, 96, 96	99	3.3	5		

Cucumber	0.01	93, 94, 91, 98, 91	93	3.1	5	95	4.2
	0.1	96, 100, 99, 99, 89	97	4.7	5		

**Table A 2: Characteristics for the analytical method used for validation of prothioconazole residues in water different matrices of plant origin**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two mass transitions per analyte. A reagent blank per analytical set and two control samples per matrix were extracted and analysed. For both mass transitions of both analytes, the samples showed no significant interference (above 30 % of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific. Representative chromatograms and product ion spectra are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Please see table A3 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.00 ng/mL (n = 5, prothioconazole-desthio), corresponding to analyte concentrations of 0.003 mg/kg to 0.2 mg/kg (prothioconazole-desthio) in matrix samples.
Assessment of matrix effects is presented	Yes (significant for oilseed rape and bean)
Limit of determination/quantification	The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for both analytes in all matrices. The limit of detection (LOD) was set at the level of the lowest calibration standard, 0.15 ng/mL for prothioconazole-desthio, equivalent to 0.003 mg/kg for prothioconazole-desthio.
Stability of standards and extracts	Calibration solutions in methanol/water (2/3, v/v) for prothioconazole and prothioconazole-desthio (0.15-10 ng/mL): stable for 10 days between 1-9°C. Extract stable stable for 10 days between 1-9°C (except for prothioconazole in bean – analysis directly after extraction recommended).

**Table A 3: Linearity of detector response**

Analyte	Matrix	Transition	Linearity data
Prothioconazole	Wheat (grain)	342 → 100	y = 269019.6651 x + 7609.2371, r = 0.9998
		342 → 125	y = 162668.0296 x + 7599.8426, r = 0.9994
	Grapes	342 → 100	y = 84468.6526 x – 331.1023, r = 0.9994
		342 → 125	y = 54979.3614 x + 1061.5103, r = 0.9995
	Oilseed rape (seeds)	342 → 100	y = 154482.6713 x – 2423.1974, r = 0.9995
		342 → 125	y = 96810.3583 x – 1013.4184, r = 0.9999
	Bean (dry)	342 → 100	y = 103352.8037 x – 470.5023, r = 0.9998
		342 → 125	y = 64989.1355 x – 191.1264, r = 0.9999
Prothioconazole-desthio	Cucumber	342 → 100	y = 250876.3629 x + 2976.5787, r = 0.9997
		342 → 125	y = 155001.3629 x + 1419.2871, r = 0.9993
	Wheat (grain)	312 → 70	y = 379803.3489 x – 3175.7042, r = 0.9998
		312 → 125	y = 199091.9003 x + 706.4512, r = 0.9999
	Grapes	312 → 70	y = 1007320.8723 x + 76813.3437, r = 0.9977
		312 → 125	y = 489156.5421 x + 23235.4945, r = 0.9976
	Oilseed rape (seeds)	312 → 70	y = 137251.9470 x + 1668.9220, r = 0.9998
		312 → 125	y = 69533.4891 x + 2055.4582, r = 0.9997
	Bean (dry)	312 → 70	y = 246299.4548 x – 3555.2148, r = 0.9998
		312 → 125	y = 128550.2336 x – 906.3960, r = 0.9997
	Cucumber	312 → 70	y = 384992.9907 x + 8337.7142, r = 0.9998
		312 → 125	y = 202167.4455 x + 2048.1244, r = 0.9998

## Conclusion

This analytical method for the determination of prothioconazole and prothioconazole-desthio content in various plant matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for prothioconazole and prothioconazole-desthio, calculated as prothioconazole-desthio in wheat (grain), grapes, oilseed rape (seeds), beans (dry) and cucumber.

### A 2.1.1.1.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.1.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### A 2.1.1.1.2 Analytical method – Determination of prothioconazole metabolites in plant matrices

#### A 2.1.1.1.2.1 Method validation

Comments of zRMS:	<p>The method has been successfully validated according to the guidance documents SANCO/825/00, rev. 8.1, SANCO/3029/99 rev. 4 of the European Commission for the determination of metabolites of prothioconazole (PTZ-<math>\alpha</math>-hydroxy-desthio, PTZ-3-, -4-, -5- and -6-hydroxy-desthio as well as the glucoside conjugates of PTZ-3-, -4- and -6-hydroxy-desthio) in wheat (whole plant, grain, straw) and oilseed rape (seed) with the LOQ of 0.01 mg/kg calculated as prothioconazole-desthio equivalents.</p> <p>All mean recoveries were in the range of 70 – 110% with relative standard deviations of <math>\leq 20\%</math> for all analytes and matrices at each level.</p> <p>The study is acceptable.</p>
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Reference: KCP 5.1.2/02

Report Validation of an Analytical Method for the Determination of relevant Metabolites of Prothioconazole in Different Matrices of Plant Origin  
Winter O., Nachtigall S., 2020, report number S16-04435 (NUD-1602V)

Guideline(s): SANCO/825/00 rev. 8.1  
SANCO/3029/99 rev. 4

Deviations: No

GLP: Yes, conducted under GLP/Officially recognised testing facilities

Acceptability: Yes

## Materials and methods

Samples of wheat (whole plant, grain, straw) and oilseed rape (seed) were extracted with acetonitrile/water (4/1, v/v) in the presence of Celite. An acidic hydrolysis under reflux was performed with the extracts using chlorhydric acid. After addition of water up to 100 mL, an aliquot (10 mL) was neutralised to pH 7 with sodium hydrogen carbonate and was cleaned by purification on a SPE cartridge (Chem Elut for wheat whole plant and grain, Chromabond Kieselgur XTR for wheat straw and oilseed

rape seeds). After elution with cyclohexane/ethyl acetate (85/15, v/v) (150 mL), the eluate was evaporated to dryness and redissolved in acetonitrile (1 mL). The extract was diluted 10 fold using acetonitrile and water. Quantification was performed by LC-MS/MS and two transitions were monitored and quantified for each analyte, please refer to the table below. In order to show the effectiveness of the hydrolysis step, glucoside conjugates of prothioconazole-3-, -4- and -6-hydroxydesthio were hydrolysed to their aglycones prothioconazole-3-, -4- and -6-hydroxy-desthio and analysed as such. This method shall be representative for incurred residues of glucoside conjugates of prothioconazole-3-, -4-, -5- and -6-hydroxy-desthio. Matrix-matched standards were used for quantitation. The intended limit of quantification (LOQ) was 0.01 mg/kg (calculated as prothioconazole-desthio) in all matrix types.

### Analytical conditions

System: 1200 Binary Rapid Resolution LC System, Agilent Technologies  
Column: Kinetex PFP 100A (100 mm x 3 mm, 2.6 µm, Phenomenex)  
Mobile phase A: Acetonitrile  
Mobile phase B: Water + 0.2 % acetic acid  
Flow: 0.7 L/min  
Column temperature: 50°C  
Injection volume: 25 µL

	A	B
0 min	20	80
6 min	30	70
8 min	90	10
9 min	90	10
9.1 min	20	80
11 min	20	80

System: API 4000 System, SCIEX (Triple quadrupole mass spectrometer)  
Ionisation type: Electrospray ionisation (ESI, TurboIon Spray)  
Polarity: Positive ion mode  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
Capillary voltage (IS) 5500 V (pos)

Analyte monitored	Mass transition monitored (m/z)
Prothioconazole- $\alpha$ -hydroxy-desthio	328 $\rightarrow$ 70 <sup>#</sup>
	328 $\rightarrow$ 141
Prothioconazole-3-hydroxy-desthio	328 $\rightarrow$ 70 <sup>#</sup>
	328 $\rightarrow$ 141
Prothioconazole-4-hydroxy-desthio	328 $\rightarrow$ 70 <sup>#</sup>
	328 $\rightarrow$ 141
Prothioconazole-5-hydroxy-desthio	328 $\rightarrow$ 70 <sup>#</sup>
	328 $\rightarrow$ 141
Prothioconazole-6-hydroxy-desthio	328 $\rightarrow$ 70 <sup>#</sup>
	328 $\rightarrow$ 141

#: used for quantification but both transitions are interchangeable.

### Results and discussions

**Table A 4: Recovery results from method validation of prothioconazole metabolites using the analytical method**

Prothioconazole- $\alpha$ -hydroxy-desthio							
Analyte: Prothioconazole- $\alpha$ -hydroxy-desthio		Final determination as: Prothioconazole- $\alpha$ -hydroxy-desthio			Residues calculated as: Prothioconazole-desthio		
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 328 $\rightarrow$ 70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	109, 107, 93, 88, 88	97	11	5	94	8.7
	0.1	97, 92, 91, 89, 85	91	4.8	5		

Wheat (grain)	0.01	110, 109, 98, 66, 92	95	19	5	91	15
	0.1	91, 89, 88, 86, 78	86	5.8	5		
Wheat (straw)	0.01	100, 93, 93, 84, 97	93	6.5	5	90	6.8
	0.1	82, 93, 86, 92, 83	87	5.8	5		
Oilseed rape (seeds)	0.01	132, 103, 114, 83, 108	108	16	5	109	13
	0.1	106, 125, 102, 115, 98	109	9.9	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	104, 101, 97, 86, 86	95	8.9	5	93	7.2
	0.1	99, 93, 91, 89, 86	92	5.3	5		
Wheat (grain)	0.01	108, 107, 99, 62, 95	94	20	5	90	15
	0.1	92, 88, 87, 85, 79	86	5.5	5		
Wheat (straw)	0.01	99, 87, 80, 79, 94	88	909	5	90	8.3
	0.1	85, 100, 91, 95, 86	91	4.9	5		
Oilseed rape (seeds)	0.01	129, 99, 112, 84, 108	106	16	5	106	12
	0.1	103, 123, 101, 110, 95	106	10	5		
Prothioconazole-3-hydroxy-desthio							
Analyte: Prothioconazole-3-hydroxy-desthio		Final determination as: Prothioconazole-3-hydroxy-desthio		Residues calculated as: Prothioconazole-desthio			
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	109, 108, 89, 78, 72	91	19	5	92	13
	0.1	103, 96, 90, 89, 87	93	7.0	5		
Wheat (grain)	0.01	107, 92, 90, 88, 81	92	10	5	91	7.2
	0.1	91, 91, 90, 88, 88	90	1.7	5		
Wheat (straw)	0.01	88, 83, 89, 79, 95	87	7.0	5	88	5.9
	0.1	87, 94, 84, 92, 84	88	5.2	5		
Oilseed rape (seeds)	0.01	107, 88, 96, 70, 79	88	16	5	94	14
	0.1	97, 110, 94, 107, 89	99	8.9	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	108, 98, 82, 76, 85	90	14	5	90	10
	0.1	97, 93, 89, 87, 85	90	5.3	5		
Wheat (grain)	0.01	104, 98, 83, 78, 80	89	13	5	89	9.0
	0.1	94, 89, 89, 88, 86	89	3.3	5		
Wheat (straw)	0.01	107, 84, 83, 99, 85	92	12	5	90	9.5
	0.1	84, 99, 88, 91, 82	89	7.5	5		
Oilseed rape (seeds)	0.01	105, 86, 94, 74, 88	89	13	5	95	12
	0.1	97, 111, 94, 109, 88	100	9.9	5		

Prothioconazole-4-hydroxy-desthio							
Analyte: Prothioconazole-4-hydroxy-desthio		Final determination as: Prothioconazole-4-hydroxy-desthio			Residues calculated as: Prothioconazole-desthio		
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	95, 90, 74, 70, 70	80	15	5	83	11
	0.1	94, 89, 84, 82, 79	86	6.9	5		
Wheat (grain)	0.01	93, 89, 81, 75, 70	82	12	5	83	8.4
	0.1	90, 84, 82, 80, 83	84	4.5	5		
Wheat (straw)	0.01	78, 71, 75, 70, 74	74	4.4	5	76	5.9
	0.1	76, 84, 76, 82, 73	78	5.9	5		
Oilseed rape (seeds)	0.01	110, 82, 99, 73, 88	90	16	5	92	13
	0.1	90, 104, 85, 104, 85	94	10	5		
Prothioconazole-4-hydroxy-desthio							
Analyte: Prothioconazole-4-hydroxy-desthio		Final determination as: Prothioconazole-4-hydroxy-desthio			Residues calculated as: Prothioconazole-desthio		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	94, 92, 79, 70, 70	81	14	5	83	11
	0.1	93, 88, 82, 83, 80	85	6.2	5		
Wheat (grain)	0.01	98, 92, 83, 74, 74	84	13	5	84	8.9
	0.1	90, 85, 83, 82, 83	85	3.8	5		
Wheat (straw)	0.01	79, 75, 76, 72, 76	76	3.3	5	76	4.5
	0.1	72, 82, 75, 78, 71	76	6.0	5		
Oilseed rape (seeds)	0.01	99, 91, 100, 75, 90	91	11	5	93	10
	0.1	88, 105, 89, 104, 86	94	9.8	5		

Prothioconazole-5-hydroxy-desthio							
Analyte: Prothioconazole-5-hydroxy-desthio		Final determination as: Prothioconazole-5-hydroxy-desthio			Residues calculated as: Prothioconazole-desthio		
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	97, 95, 80, 75, 70	83	14	5	85	10
	0.1	94, 84, 85, 85, 81	86	5.7	5		
Wheat (grain)	0.01	104, 97, 87, 87, 79	91	11	5	90	7.5
	0.1	93, 90, 89, 86, 89	89	2.8	5		
Wheat (straw)	0.01	84, 76, 78, 70, 83	78	7.3	5	80	6.3
	0.1	81, 87, 79, 86, 79	82	4.7	5		

Oilseed rape (seeds)	0.01	105, 87, 98, 70, 94	91	15	5	92	11
	0.1	88, 101, 88, 102, 87	93	8.2	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	106, 95, 88, 72, 68	86	18	5	86	13
	0.1	97, 85, 83, 84, 81	86	7.4	5		
Wheat (grain)	0.01	103, 103, 92, 86, 79	93	11	5	90	8.4
	0.1	92, 89, 87, 85, 88	88	2.9	5		
Wheat (straw)	0.01	90, 84, 89, 72, 76	82	9.7	5	79	8.7
	0.1	71, 80, 79, 76, 72	76	5.3	5		
Oilseed rape (seeds)	0.01	105, 74, 99, 81, 86	89	14	5	90	12
	0.1	87, 102, 83, 97, 81	90	10	5		

Prothioconazole-6-hydroxy-desthio							
Analyte: Prothioconazole-6-hydroxy-desthio		Final determination as: Prothioconazole-6-hydroxy-desthio			Residues calculated as: Prothioconazole-desthio		
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	86, 86, 72, 70, 66	76	12	5	78	9.7
	0.1	89, 80, 77, 75, 75	79	7.4	5		
Wheat (grain)	0.01	100, 92, 89, 81, 77	88	10	5	87	7.2
	0.1	89, 87, 86, 86, 83	86	2.5	5		
Wheat (straw)	0.01	71, 70, 73, 66, 72	70	3.8	5	71	5.1
	0.1	71, 78, 70, 76, 67	72	6.2	5		
Oilseed rape (seeds)	0.01	94, 79, 77, 68, 70	78	13	5	80	11
	0.1	80, 91, 76, 90, 75	82	9.3	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	86, 83, 74, 68, 66	75	12	5	78	9.8
	0.1	90, 81, 78, 77, 74	80	7.7	5		
Wheat (grain)	0.01	101, 95, 90, 84, 77	89	10	5	88	7.4
	0.1	88, 86, 86, 86, 85	86	1.3	5		
Wheat (straw)	0.01	71, 73, 74, 68, 70	71	3.4	5	73	4.8
	0.1	74, 80, 74, 77, 71	75	4.5	5		
Oilseed rape (seeds)	0.01	92, 72, 77, 68, 76	77	12	5	80	12
	0.1	82, 92, 75, 94, 74	83	11	5		

<b>Prothioconazole-desthio-3-glucoside</b>			
Analyte: Prothioconazole- desthio-3-glucoside		Final determination as: Prothioconazole- desthio-3-glucoside	
		Residues calculated as: Prothioconazole-desthio	

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	96, 98, 90, 96, 85	93	5.8	5	93	5.8
	0.1	102, 92, 93, 88, 87	92	6.4	5		
Wheat (grain)	0.01	87, 87, 88, 86, 85	87	1.3	5	85	8.0
	0.1	91, 85, 67, 81, 89	83	12	5		
Wheat (straw)	0.01	85, 85, 78, 81, 85	83	3.9	5	86	5.2
	0.1	92, 88, 91, 89, 90	90	1.8	5		
Oilseed rape (seeds)	0.01	84, 87, 84, 88, 78	84	4.6	5	81	7.5
	0.1	84, 84, 77, 73, 70	78	8.2	5		
Prothioconazole-desthio-3-glucoside							
Analyte: Prothioconazole- desthio-3-glucoside		Final determination as: Prothioconazole- desthio-3-glucoside		Residues calculated as: Prothioconazole-desthio			
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	100, 102, 88, 82, 84	91	10	5	91	8.5
	0.1	102, 94, 88, 88, 84	91	7.7	5		
Wheat (grain)	0.01	79, 92, 88, 83, 90	86	6.2	5	85	8.1
	0.1	91, 89, 70, 80, 87	83	10	5		
Wheat (straw)	0.01	84, 86, 86, 75, 92	85	7.3	5	87	6.2
	0.1	93, 89, 91, 85, 92	90	3.5	5		
Oilseed rape (seeds)	0.01	87, 86, 84, 94, 91	88	4.6	5	83	9.8
	0.1	82, 85, 74, 72, 70	77	8.5	5		
Prothioconazole-desthio-4-glucoside							
Analyte: Prothioconazole- desthio-4-glucoside		Final determination as: Prothioconazole- desthio-4-glucoside		Residues calculated as: Prothioconazole-desthio			
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	91, 86, 80, 80, 74	82	7.9	5	84	7.8
	0.1	96, 86, 84, 80, 78	85	8.3	5		
Wheat (grain)	0.01	78, 76, 80, 78, 81	79	2.5	5	77	7.6
	0.1	82, 80, 62, 75, 82	76	11	5		
Wheat (straw)	0.01	82, 81, 81, 78, 84	81	2.7	5	88	8.3
	0.1	95, 90, 98, 94, 94	94	3.0	5		
Oilseed rape (seeds)	0.01	94, 92, 83, 92, 85	89	5.5	5	86	7.2
	0.1	90, 89, 83, 78, 76	83	7.6	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							

Wheat (whole plant)	0.01	90, 85, 79, 78, 70	80	9.4	5	84	9.0
	0.1	96, 91, 86, 83, 80	87	7.3	5		
Wheat (grain)	0.01	75, 73, 75, 73, 76	74	1.8	5	75	7.4
	0.1	83, 80, 63, 70, 78	75	11	5		
Wheat (straw)	0.01	87, 89, 76, 81, 80	83	6.4	5	89	8.5
	0.1	97, 92, 97, 95, 94	95	2.2	5		
Oilseed rape (seeds)	0.01	80, 85, 84, 78, 82	82	3.5	5	82	6.2
	0.1	89, 89, 80, 76, 74	82	8.7	5		

Prothioconazole-desthio-6-glucoside							
Analyte: Prothioconazole- desthio-6-glucoside		Final determination as: Prothioconazole- desthio-6-glucoside			Residues calculated as: Prothioconazole-desthio		
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328→70 (Proposed for Quantification)							
Wheat (whole plant)	0.01	87, 90, 82, 90, 82	86	4.7	5	86	5.5
	0.1	93, 86, 86, 80, 79	85	6.6	5		
Wheat (grain)	0.01	89, 88, 87, 84, 88	87	2.2	5	84	6.8
	0.1	86, 84, 70, 79, 85	81	8.2	5		
Wheat (straw)	0.01	84, 73, 79, 81, 87	81	6.6	5	82	5.5
	0.1	88, 83, 85, 79, 85	84	3.9	5		
Oilseed rape (seeds)	0.01	80, 70, 73, 74, 73	74	5.0	5	74	5.7
	0.1	82, 78, 74, 70, 70	75	7.0	5		
Transition <i>m/z</i> 328→141 (Proposed for Confirmation)							
Wheat (whole plant)	0.01	87, 90, 78, 84, 79	86	4.7	5	85	5.8
	0.1	93, 86, 86, 82, 80	85	6.6	5		
Wheat (grain)	0.01	86, 89, 86, 81, 87	87	2.2	5	83	6.2
	0.1	86, 84, 72, 77, 84	81	8.2	5		
Wheat (straw)	0.01	76, 81, 79, 87, 82	81	6.6	5	83	4.7
	0.1	89, 82, 86, 82, 85	84	3.9	5		
Oilseed rape (seeds)	0.01	89, 83, 77, 80, 81	74	5.0	5	78	8.0
	0.1	82, 78, 74, 70, 68	75	7.0	5		

**Table A 5: Characteristics for the analytical method used for validation of residues of prothioconazole metabolites in plant matrices**

	Prothioconazole metabolites
Specificity	MS/MS determination was conducted by monitoring two mass transitions per analyte. A reagent blank per analytical set and two control samples per matrix were extracted and analysed. For both mass transitions of both analytes, the samples showed no significant interference (above 30% of LOQ) at the retention time of the analytes in any investigated matrix, therefore showing that the method is highly specific. Representative chromatograms and product ion spectra are provided.
Calibration (type,	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear

	Prothioconazole metabolites
number of data points)	calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Please see table A6 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.00 ng/mL (n = 7), corresponding to analyte concentrations of 0.003 mg/kg to 0.2 mg/kg expressed as prothioconazole-desthio in matrix samples.
Assessment of matrix effects is presented	Yes for prothioconazole - $\alpha$ -hydroxy-desthio, prothioconazole -3-hydroxy-desthio, prothioconazole -4-hydroxy-desthio, prothioconazole -5-hydroxy-desthio and prothioconazole -6-hydroxy-desthio (significant for at least one analyte except for wheat grain)
Limit of determination/quantification	The LOQ is the lowest validated fortification level for prothioconazole- $\alpha$ -hydroxy-desthio, prothioconazole-3-, -4-, -5- and -6-hydroxy-desthio as well as the glucoside conjugates of prothioconazole-3-, -4- and -6-hydroxy-desthio and was thus successfully established at 0.01 mg/kg calculated in prothioconazole-desthio equivalents in wheat (whole plant, grain, straw) and oilseed rape (seed) for the two mass transitions. The LOD was set at 0.003 mg/kg, which is 30 % of the LOQ.
Stability of standards and extracts	Calibration solutions in acetonitrile for prothioconazole - $\alpha$ -hydroxy-desthio, prothioconazole -3-hydroxy-desthio, prothioconazole -4-hydroxy-desthio, prothioconazole -5-hydroxy-desthio and prothioconazole -6-hydroxy-desthio (50 ng/mL): stable for 20 days between 1-10°C. Extract stable stable between 1-10°C: wheat (whole plant) for 20 days, wheat (grain) for 17 days, wheat (straw) for 14 days oilseed rape (seed) for 23 days

**Table A 6: Linearity of detector response**

Analyte	Matrix	Transition (m/z)	Linearity data
Prothioconazole- $\alpha$ -hydroxy-desthio	Wheat (whole plant)	328→70	y = 34319.0296 x + 4179.6946, r = 0.9988
		328→141	y = 11376.1941 x + 1492.0611, r = 0.9988
	Wheat (grain)	328→70	y = 35976.0930 x + 2192.4459, r = 0.9990
		328→141	y = 11805.7114 x + 916.8273, r = 0.9989
	Wheat (straw)	328→70	y = 14862.5221 x + 391.9694, r = 0.9995
		328→141	y = 4798.1804 x + 458.3559, r = 0.9986
	Oilseed rape (seeds)	328→70	y = 15828.8097 x + 2350.3173, r = 0.9997
		328→141	y = 5148.3952 x + 817.8952, r = 0.9996
Prothioconazole-3-hydroxy-desthio	Wheat (whole plant)	328→70	y = 32515.6432 x + 6074.0157, r = 0.9968
		328→141	y = 12912.6106 x + 2319.8469, r = 0.9978
	Wheat (grain)	328→70	y = 39927.3187 x + 4616.7082, r = 0.9983
		328→141	y = 15333.6871 x + 1641.0340, r = 0.9974
	Wheat (straw)	328→70	y = 14741.4708 x + 448.5433, r = 0.9998
		328→141	y = 5473.5911 x + 428.3996, r = 0.9991
	Oilseed rape (seeds)	328→70	y = 14084.0030 + 2883.0456, r = 0.9994
		328→141	y = 5331.0083 x + 946.9468, r = 0.9993
Prothioconazole-4-hydroxy-desthio	Wheat (whole plant)	328→70	y = 43145.8175 x + 10394.8518, r = 0.9945
		328→141	y = 30558.1501 x + 7170.7571, r = 0.9955
	Wheat (grain)	328→70	y = 54104.1193 x + 8317.8887, r = 0.9954
		328→141	y = 37628.3043 x + 5606.5273, r = 0.9959
	Wheat (straw)	328→70	y = 18411.0184 + 1480.9083, r = 0.9993
		328→141	y = 12737.6801 x + 910.1181, r = 0.9993
	Oilseed rape (seeds)	328→70	y = 12878.8982 x + 1661.0520, r = 0.9993
		328→141	y = 8913.7731 x + 1108.2783, r = 0.9994
Prothioconazole-5-hydroxy-desthio	Wheat (whole plant)	328→70	y = 35861.0564 x + 6127.5497, r = 0.9978
		328→141	y = 14577.8115 x + 2680.1892, r = 0.9980
	Wheat (grain)	328→70	y = 46383.7756 x + 5591.7687, r = 0.9966
		328→141	y = 18779.3278 x + 2134.4164, r = 0.9974

Analyte	Matrix	Transition (m/z)	Linearity data
	Wheat (straw)	328→70	$y = 16392.2669 x + 1165.1554, r = 0.9997$
		328→141	$y = 6674.6020 x + 278.8368, r = 0.9988$
	Oilseed rape (seeds)	328→70	$y = 12019.7624 x + 1860.4758, r = 0.9998$
		328→141	$y = 4842.1531 x + 1085.2312, r = 0.9999$
Prothioconazole- $\alpha$ -hydroxy-desthio	Wheat (whole plant)	328→70	$y = 30833.2070 x + 4869.2906, r = 0.9978$
		328→141	$y = 17229.6184 x + 3078.6671, r = 0.9972$
	Wheat (grain)	328→70	$y = 45060.8542 x + 4962.6051, r = 0.9964$
		328→141	$y = 25106.3432 x + 2617.9934, r = 0.9957$
	Wheat (straw)	328→70	$y = 14531.6654 x + 740.1596, r = 0.9996$
		328→141	$y = 7981.6022 x - 144.2428, r = 0.9996$
	Oilseed rape (seeds)	328→70	$y = 9314.7839 x + 1067.2869, r = 0.9994$
		328→141	$y = 4951.9838 x + 728.5187, r = 0.9992$
Prothioconazole-desthio-3-glucoside	Wheat (whole plant)	328→70	$y = 42087.9454 x + 5922.3221, r = 0.9980$
		328→141	$y = 16480.3134 x + 2375.6641, r = 0.9980$
	Wheat (grain)	328→70	$y = 44340.7127 x + 687.1439, r = 0.9996$
		328→141	$y = 17191.7109 x + 225.8435, r = 0.9995$
	Wheat (straw)	328→70	$y = 5024.8168 x + 526.9475, r = 0.9998$
		328→141	$y = 6028.1021 x + 287.2970, r = 1.0000$
	Oilseed rape (seeds)	328→70	$y = 4299.5906 x + 148.8444, r = 0.9991$
		328→141	$y = 5455.3247 x - 60.0578, r = 0.9989$
Prothioconazole-desthio-4-glucoside	Wheat (whole plant)	328→70	$y = 51710.8921 x + 10386.3894, r = 0.9948$
		328→141	$y = 36036.8966 x + 7330.9578, r = 0.9960$
	Wheat (grain)	328→70	$y = 54948.7996 + 2627.7844, r = 0.9991$
		328→141	$y = 39813.3940 x + 1984.7215, r = 0.9992$
	Wheat (straw)	328→70	$y = 5503.5633 x + 284.2911, r = 0.9999$
		328→141	$y = 12040.4347 x + 434.6309, r = 1.0000$
	Oilseed rape (seeds)	328→70	$y = 4144.6753 x + 88.6292, r = 0.9994$
		328→141	$y = 9276.9270 x + 235.6995, r = 0.9990$
Prothioconazole-desthio-6-glucoside	Wheat (whole plant)	328→70	$y = 47155.9262 x + 5442.0809, r = 0.9973$
		328→141	$y = 26691.4834 x + 3243.1380, r = 0.9977$
	Wheat (grain)	328→70	$y = 69386.9093 x + 634.1240, r = 0.9996$
		328→141	$y = 39042.1026 x + 292.5665, r = 0.9998$
	Wheat (straw)	328→70	$y = 5724.8218 x + 33.9283, r = 0.9997$
		328→141	$y = 9935.8100 x + 267.2378, r = 0.9999$
	Oilseed rape (seeds)	328→70	$y = 3990.5181 x + 58.3848, r = 0.9994$
		328→141	$y = 7189.8913 x - 52.9434, r = 0.9996$

## Conclusion

This analytical method for the determination of prothioconazole- $\alpha$ -hydroxy-desthio, prothioconazole3-, -4-, -5- and -6-hydroxy-desthio as well as the glucoside conjugates of prothioconazole-3-, -4- and -6-hydroxy-desthio content in various plant matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for Prothioconazole- $\alpha$ -hydroxy-desthio, prothioconazole3-, -4-, -5- and -6-hydroxy-desthio as well as the glucoside conjugates of prothioconazole-3-, -4- and -6-hydroxy-desthio, calculated as prothioconazole in wheat (whole plant, grain and straw) and oilseed rape (seeds).

#### A 2.1.1.1.2.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

#### A 2.1.1.1.2.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

#### A 2.1.1.2 Analytical method – Determination of triazole derivative metabolites in plant matrices

##### A 2.1.1.2.1.1 Method validation 1

Comments of zRMS:	<p>The method has been successfully validated according to the guidance document SANCO/825/00, rev. 8.1 for the determination of 1,2,4-triazole (T) and the triazole-based metabolites, triazole alanine (TA), triazole lactic acid (TLA) and triazole acetic acid (TAA) in wheat (grain and straw), barley (grain and straw), grape (bunches) and oilseed rape with the LOQ of 0.01 mg/kg.</p> <p>All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20 % for all analytes and matrices at each level.</p> <p>The study is acceptable.</p>
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Reference: KCP 5.1.2/03

Report Validation of an Analytical Method for the Determination of Triazole and Triazole-based Metabolites in the Agricultural Commodity Wheat, Barley, Grape and Rape  
Schernikau N., 2016, report No. S15-03542 (GAB-1537V)

Guideline(s): SANCO/825/00 rev. 8.1  
ENV/JM/MONO(2007)17, OECD

Deviations: No

GLP: Yes, conducted under GLP/Officially recognised testing facilities

Acceptability: Yes

#### Materials and methods

This analytical method was developed for the determination of 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in various plant materials with a limit of quantitation (LOQ) of 0.01 mg/kg per analyte. The analytes are extracted from the plant matrix (wheat grain and straw, barley grain and straw, grape and oilseed rape seeds – 5 g) with methanol/water (4/1 v/v) (60 mL). An aliquot (6 mL) is filtered, concentrated by evaporation and re-dissolved in water (to 5 mL). Internal standard solution (0.5 mL at 80 ng/mL) was added to the extracts prior evaporation. Extracts are analysed by liquid chromatography using differential mobility and triple-quadrupole mass spectrometry (LC-DMS/MS/MS). Solvent calibration standards with internal standards were used for quantitation.

#### Analytical conditions

System: Series 1290 HPLC, Agilent Technologies  
Pre-column: SecurityGuard™ for C18 HPLC,  
Phenomenex  
Column: Thermo Hypercarb 100x3 mm, 5.0 µm  
Mobile phase A: Methanol 0.5% (v/v) formic acid

Mobile phase B: Water + 0.5 % (v/v)  
formic acid  
Flow: 0.7 mL/min  
Column temperature: 60°C  
Injection volume: 20 µL

	A	B
0 min	0	100
3 min	0	100

6.5 min	80	20
6.51 min	0	100
9 min	0	100

### Analytical conditions for confirmation

System: Series 1290 HPLC, Agilent Technologies  
Pre-column: SecurityGuard™ for C18 HPLC, Phenomenex  
Column: Synergi 4u Polar-RP 80A, 150x4.6 mm, 4.0 µm  
Mobile phase A: Methanol 0.5% (v/v) formic acid  
Mobile phase B: Water + 0.5 % (v/v) formic acid  
Flow: 0.8 mL/min  
Column temperature: 40°C  
Injection volume: 30 µL

	A	B
0 min	30	70
4 min	80	20
4.01 min	30	70
8 min	30	70

System: Triple Quad 6500 Mass spectrometer, Applied Biosystems equipped with DMS SelexION technology  
Ionisation type: Electrospray ionisation (ESI, TurboIon Spray)  
Polarity: Positive ion mode  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
Capillary voltage (IS) 5500 V (pos)

The following mass transitions were monitored:

Analyte monitored	Mass transition monitored (m/z)
1,2,4-T	70 → 43*
1,2,4-T ISTD	75 → 46
TA	157 → 70*
	157 → 88
TA ISTD	159 → 75
TAA	128 → 70*
TAA ISTD	133 → 75
TLA	158 → 70*
TLA ISTD	163 → 75

\* Quantitation transition

### Results and discussions

Accuracy was determined by fortification of control samples with known amounts of the reference items and subsequent determination of the recoveries when applying the extraction procedure. Precision was determined by repeatability (relative standard deviation). The triazole metabolites were spiked together and quantified separately. The recovery values are corrected for corresponding mean control residue.

**Table A 7: Recovery results from method validation of triazole derivative metabolites using the analytical method**

1,2,4-Triazole							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 70→43 (Proposed for Quantification)							
Wheat (grain)	0.01	107, 99, 107, 113, 98	105	6.0	5	104	5.1
	0.1	106, 106, 95, 103, 102	102	4.4	5		
Wheat (straw)	0.01	108, 114, 110, 76, 115	105	16	5	103	12
	0.1	103, 99, 89, 114, 97	100	9.1	5		
Barley (grain)	0.01	102, 104, 101, 99, 80	97	10	5	97	8.0
	0.1	89, 104, 104, 97, 94	98	6.7	5		
Barley (straw)	0.01	105, 107, 108, 110, 107	107	1.7	5	105	4.8
	0.1	99, 102, 111, 107, 95	103	6.2	5		
Grape (bunches)	0.01	105, 102, 90, 100, 107	101	6.6	5	101	6.5
	0.1	89, 102, 105, 107, 105	102	7.2	5		
Oilseed rape	0.01	86, 87, 85, 98, 73	86	10	5	85	8.2
	0.1	81, 79, 87, 90, 79	83	6.0	5		
Mass Transition 70→43 <i>m/z</i> (confirmation method)							
Wheat (grain)	0.01	112, 97, 109, 108, 117	109	6.8	5	106	6.1
	0.1	109, 104, 103, 99, 99	103	4.0	5		
Wheat (straw)	0.01	103, 103, 95, 85, 83	94	10	5	99	9.7
	0.1	94, 113, 100, 107, 106	104	7.0	5		
Barley (grain)	0.01	108, 106, 88, 111, 113	105	9.5	5	105	6.9
	0.1	108, 108, 102, 110, 100	106	4.1	5		
Barley (straw)	0.01	105, 100, 113, 105, 97	104	5.8	5	105	6.9
	0.1	112, 101, 115, 93, 109	106	8.4	5		
Grape (bunches)	0.01	95, 100, 87, 102, 100	97	6.3	5	101	6.6
	0.1	103, 101, 110, 103, 110	105	4.1	5		
Oilseed rape	0.01	92, 90, 77, 55, 74	78	19	5	79	13
	0.1	79, 85, 81, 79, 76	80	4.1	5		

Triazole alanine							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 157→70 (Proposed for Quantification)							
Wheat (grain)	0.01	86, 104, 105, 87, 80	92	12	5	95	13
	0.1	78, 96, 107, 115, 91	97	15	5		
Wheat (straw)	0.01	96, 93, 91, 98, 93	94	2.9	5	88	11
	0.1	73, 85, 70, 95, 82	81	12	5		
Barley (grain)	0.01	78, 105, 92, 106, 74	91	16	5	93	14
	0.1	90, 111, 77, 98, 100	95	13	5		
Barley (straw)	0.01	86, 95, 94, 108, 91	95	8.6	5	96	10
	0.1	96, 98, 114, 80, 100	98	12	5		
Grape (bunches)	0.01	103, 110, 96, 105, 108	104	5.2	5	104	6.0
	0.1	106, 108, 106, 107, 90	103	7.3	5		
Oilseed rape	0.01	97, 73, 105, 97, 85	91	14	5	97	15
	0.1	98, 98, 80, 120, 113	102	15	5		
Mass Transition 157→88 <i>m/z</i> (confirmation)							
Wheat (grain)	0.01	74, 100, 104, 91, 97	93	13	5	98	12
	0.1	93, 113, 106, 109, 88	102	11	5		
Wheat (straw)	0.01	99, 102, 115, 97, 98	102	7.2	5	92	14
	0.1	79, 92, 70, 87, 82	82	10	5		
Barley (grain)	0.01	69, 110, 99, 105, 93	95	17	5	94	15
	0.1	96, 104, 70, 88, 105	93	16	5		
Barley (straw)	0.01	102, 88, 95, 112, 97	99	9.0	5	98	10
	0.1	93, 96, 113, 79, 101	96	13	5		
Grape (bunches)	0.01	90, 106, 97, 81, 106	96	11	5	97	8.7
	0.1	90, 106, 97, 81, 106	98	6.5	5		
Oilseed rape	0.01	114, 93, 115, 115, 91	106	12	5	100	13
	0.1	93, 82, 82, 110, 108	95	14	5		

Triazole lactic acid							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 158→70 (Proposed for Quantification)							
Wheat (grain)	0.01	100, 88, 86, 91, 92	91	5.9	5	87	7.4
	0.1	91, 81, 79, 85, 80	83	5.9	5		
Wheat (straw)	0.01	108, 100, 100, 93, 98	100	5.4	5	101	6.5
	0.1	101, 90, 98, 112, 105	101	8.1	5		
Barley (grain)	0.01	93, 84, 84, 86, 71	84	9.5	5	89	9.6
	0.1	91, 89, 95, 103, 94	94	5.7	5		
Barley (straw)	0.01	109, 107, 116, 110, 107	110	3.4	5	108	4.4
	0.1	101, 109, 110, 99, 108	105	4.8	5		
Grape (bunches)	0.01	100, 105, 109, 99, 110	105	4.8	5	104	4.7
	0.1	95, 104, 101, 108, 107	103	5.1	5		
Oilseed rape	0.01	105, 98, 108, 102, 111	105	4.8	5	105	3.6
	0.1	106, 103, 109, 107, 104	106	2.3	5		
Mass Transition 158→70 <i>m/z</i> (confirmation method)							
Wheat (grain)	0.01	84, 103, 87, 92, 97	93	8.2	5	92	6.1
	0.1	96, 89, 88, 90, 91	91	3.4	5		
Wheat (straw)	0.01	110, 103, 93, 96, 95	99	7.1	5	97	5.9
	0.1	98, 96, 89, 97, 96	95	3.7	5		
Barley (grain)	0.01	102, 81, 96, 96, 106	96	9.9	5	95	8.1
	0.1	87, 92, 88, 101, 96	93	6.3	5		
Barley (straw)	0.01	99, 94, 98, 104, 109	101	5.8	5	103	4.8
	0.1	103, 103, 106, 101, 110	105	3.4	5		
Grape (bunches)	0.01	106, 108, 98, 102, 113	105	5.4	5	106	3.9
	0.1	102, 107, 108, 106, 107	106	2.2	5		
Oilseed rape	0.01	108, 107, 107, 102, 108	106	2.4	5	106	2.3
	0.1	103, 105, 109, 103, 107	105	2.5	5		

Triazole acetic acid							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 128→70 (Proposed for Quantification)							
Wheat (grain)	0.01	92, 106, 103, 114, 95	102	8.6	5	98	9.2
	0.1	97, 88, 83, 100, 97	93	7.7	5		
Wheat (straw)	0.01	102, 102, 102, 98, 107	102	3.1	5	104	4.4
	0.1	108, 113, 99, 107, 102	106	5.2	5		
Barley (grain)	0.01	77, 89, 67, 88, 82	81	11	5	88	12
	0.1	91, 103, 93, 92, 99	96	5.4	5		
Barley (straw)	0.01	102, 108, 109, 103, 110	106	3.4	5	105	5.1
	0.1	102, 108, 114, 99, 97	104	6.7	5		
Grape (bunches)	0.01	108, 106, 104, 109, 108	107	1.9	5	106	2.8
	0.1	99, 108, 108, 108, 106	106	3.7	5		
Oilseed rape	0.01	102, 106, 106, 103, 106	105	1.9	5	103	4.0
	0.1	104, 94, 99, 106, 99	100	4.7	5		
Mass Transition 128→70 <i>m/z</i> (confirmation method)							
Wheat (grain)	0.01	82, 102, 101, 110, 112	101	12	5	96	11
	0.1	95, 87, 86, 88, 96	90	5.2	5		
Wheat (straw)	0.01	106, 107, 86, 99, 110	102	9.5	5	101	9.0
	0.1	96, 95, 91, 113, 110	101	9.7	5		
Barley (grain)	0.01	90, 82, 76, 88, 73	82	9.0	5	87	8.9
	0.1	90, 97, 92, 94, 86	92	4.5	5		
Barley (straw)	0.01	106, 102, 106, 105, 106	105	1.6	5	105	2.4
	0.1	102, 108, 107, 100, 104	104	3.2	5		
Grape (bunches)	0.01	93, 107, 104, 103, 107	103	5.6	5	104	4.2
	0.1	103, 109, 105, 103, 103	105	2.5	5		
Oilseed rape	0.01	101, 112, 118, 100, 106	107	7.1	5	107	6.1
	0.1	111, 97, 109, 104, 112	107	5.8	5		

**Table A 8: Characteristics for the analytical method used for validation of triazole derivative metabolite residues in plant matrices**

	Triazole derivative metabolites
Specificity	Quantification was performed by use of LC-MS/MS detection and by LC-DMS-MS/MS. Two selected mass transitions were evaluated for TA in order to demonstrate that the method achieves a high level of selectivity. For 1,2,4-T, TAA and TLA two characteristic mass transitions were not available, therefore an independent analytical technique was applied for confirmation purposes. The extracts were injected twice by using HPLC columns of different stationary phases. Due to the presence of triazole metabolite residues in the control samples, recoveries were corrected for the amount of triazoles. Based on the chromatograms it can be concluded that the method was proven to be selective for each of the analytes considered. For each analyte two mass transitions were evaluated successfully. Representative chromatograms and product ion spectra are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Please see table below.
Calibration range	Linearity was confirmed over the calibration range 0.2 – 20 ng/mL (n = 8) corresponding to analyte concentrations of 0.002 mg/kg to 0.2 mg/kg in matrix samples.
Assessment of matrix effects is presented	No, the internal standard procedure, using stable isotopically labelled internal standards, compensates for matrix effects.
Limit of determination/quantification	The limit of quantification (LOQ) is the lowest validated fortification level for each analyte and was thus successfully established at 0.01 mg/kg in wheat (grain and straw), barley (grain and straw) grape (bunches) and oilseed rape for both mass transitions of each analyte. The LOD was set at 0.003 mg/kg for all matrices, which is 30 % of the LOQ. Following the publication of guidance document SANTE/2020/12830 rev.1, the LOD is now defined as the level of the lowest calibration standard, 0.2 ng/mL, which is equivalent to 0.002 mg/kg.
Stability of standards and extracts	Stock solutions in water for 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) and their radiolabelled internal standards (20 ng/mL): stable for at least 2 months < -18°C. Calibration solutions in water for 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) (1-30 ng/mL): stable for 10 days < -18°C. Extract: not assessed as stable isotopically labelled internal standards are used.

**Table A 9: Linearity of detector response**

Analyte	Matrix	Transition (m/z)	Linearity data
1,2,4-T	Wheat (grain)	70→43	$y = 0.1053 x + 0.0071, r = 0.9978$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1090 x + 0.0032, r = 0.9994$
		75→46 (ISTD)	
	Wheat (straw)	70→43	$y = 0.0980 x + 0.0046, r = 0.9961$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1101 x + 0.0027, r = 0.9997$
		75→46 (ISTD)	
	Barley (grain)	70→43	$y = 0.1231 x - 0.0010, r = 0.9992$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1104 x - 0.0038, r = 0.9992$
		75→46 (ISTD)	
	Barley (straw)	70→43	$y = 0.1108 x - 0.0002, r = 0.9990$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1219 x - 0.0001, r = 0.9995$
		75→46 (ISTD)	
	Grapes	70→43	$y = 0.1094 x - 0.0018, r = 0.9992$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1101 x + 0.0027, r = 0.9997$
		75→46 (ISTD)	
	Oilseed rape	70→43	$y = 0.0980 x + 0.0046, r = 0.9961$
		75→46 (ISTD)	
		70→43	Confirmatory method $y = 0.1101 x + 0.0027, r = 0.9997$
		75→46 (ISTD)	
TA	Wheat (grain)	157→70	$y = 0.1909 x + 0.0044, r = 0.9973$
		162→75 (ISTD)	
		157→88	$y = 0.0862 x + 0.0017, r = 0.9969$
		162→75 (ISTD)	
	Wheat (straw)	157→70	$y = 0.2028 x + 0.0135, r = 0.9992$
		162→75 (ISTD)	
		157→88	$y = 0.0932 x + 0.0050, r = 0.9995$
		162→75 (ISTD)	
	Barley (grain)	157→70	$y = 0.1796 x + 0.0185, r = 0.9982$
		162→75 (ISTD)	
		157→88	$y = 0.0788 x + 0.0051, r = 0.9992$
		162→75 (ISTD)	
	Barley (straw)	157→70	$y = 0.2094 x + 0.0105, r = 0.9959$
		162→75 (ISTD)	
		157→88	$y = 0.0920 x + 0.0044, r = 0.9974$
		162→75 (ISTD)	
	Grapes	157→70	$y = 0.2028 x + 0.0135, r = 0.9992$
		162→75 (ISTD)	
		157→88	$y = 0.0932 x + 0.0050, r = 0.9995$
		162→75 (ISTD)	
	Oilseed rape	157→70	$y = 0.1793 x + 0.0270, r = 0.9914$
		162→75 (ISTD)	
		157→88	$y = 0.0808 x + 0.0121, r = 0.9885$
		162→75 (ISTD)	
Analyte	Matrix	Transition (m/z)	Linearity data
TLA	Wheat (grain)	158→70	$y = 0.1208 x - 0.0001, r = 0.9961$
		163→75 (ISTD)	
		158→70	Confirmatory method

		163→75 (ISTD)	$y = 0.1085 x + 0.0050, r = 0.9995$
	Wheat (straw)	158→70 163→75 (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 163→75 (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9968$
	Barley (grain)	158→70 163→75 (ISTD)	$y = 0.1215 x + 0.0146, r = 0.9977$
		158→70 163→75 (ISTD)	Confirmatory method $y = 0.1558 x - 0.0035, r = 0.9993$
	Barley (straw)	158→70 163→75 (ISTD)	$y = 0.1199 x + 0.0001, r = 0.9995$
		158→70 163→75 (ISTD)	Confirmatory method $y = 0.1160 x + 0.0015, r = 0.9997$
	Grapes	158→70 163→75 (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 163→75 (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9988$
	Oilseed rape	158→70 163→75 (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 163→75 (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9988$
TAA	Wheat (grain)	128→70 133→75 ISTD	$y = 0.1325 x + 0.0039, r = 0.9961$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1230 x + 0.0097, r = 0.9989$
	Wheat (straw)	128→70 133→75 ISTD	$y = 0.1243 x + 0.00668, r = 0.9978$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1262 x + 0.0032, r = 0.9992$
	Barley (grain)	128→70 133→75 ISTD	$y = 0.1205 x + 0.0077, r = 0.9993$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1297 x - 0.0015, r = 0.9998$
	Barley (straw)	128→70 133→75 ISTD	$y = 0.1342 x + 0.0033, r = 0.9996$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1311 x + 0.0034, r = 0.9996$
	Grapes	128→70 133→75 ISTD	$y = 0.1243 x + 0.0068, r = 0.9978$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1262 x + 0.0032, r = 0.9992$
	Oilseed rape	128→70 133→75 ISTD	$y = 0.1229 x + 0.0079, r = 0.9979$
		128→70 133→75 ISTD	Confirmatory method $y = 0.1262 x + 0.0032, r = 0.9992$

## Conclusion

This analytical method for the determination of 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid content in various plant matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 and SANCO/3029/99 rev.4 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for triazole derivative metabolites in wheat (grain and straw), barley (grain and straw) grape (bunches) and oilseed rape.

### A 2.1.1.2.1.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.1.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change

of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

#### A 2.1.1.2.1.4 Method validation 2

Comments of zRMS:	<p>The analytical BCS method 01062/M004 has been successfully validated for the determination of 1,2,4-triazole (T), triazolylalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in various plant materials (five different crop types (full validation sets performed) and in many additional plants matrices (reduced validation sets performed) by LC/DMS/MS/MS using stable isotopically labelled internal standards with LOQ of 0.01 mg/kg.</p> <p>Mean recoveries were within the 70 - 110% range for most matrix (16) and analyte (4) combinations. Few average recoveries were above 110% but &lt; 120%, obviously caused by analyte present in control samples requiring background correction and supported by acceptable relative standard deviations (RSDs) and thus considered acceptable.</p> <p>Relative standard deviations were below 20% for all analytes and sample materials fortified at 0.01 mg/kg (LOQ), except for triazolylalanine (TA) in sunflower seed, melon peel and melon pulp (RSDs &lt; 30%, caused by endogenous TA present in the untreated sample requiring background subtraction. Nevertheless, these results are considered acceptable.</p> <p>The method meets in general all guideline criteria and is acceptable.</p>
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Reference: KCP 5.1.2/04

Report Modification M004 of BCS residue analytical method 01062 for the determination of 1,2,4-Triazole, Triazolylalanine, Triazole acetic acid and Triazole lactic acid by LC/DMS/MS/MS in plant materials  
Class, T., 2011, Method 01062/M004, Report No. P 2383G, M-420638-01-1

Guideline(s): ENV/JM/MONO(2007)17  
SANCO/3029/99 rev.4 (11/07/2000)

Deviations: -

GLP: Yes, conducted under GLP/Officially recognised testing facilities

Acceptability: Yes

#### Materials and methods

The analytical method 0170/02 (BCS 01062/M004) was developed for the determination of 1,2,4-triazole (T), triazolylalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in various plant materials with a limit of quantitation (LOQ) of 0.01 mg/kg per analyte. The analytes are extracted from crop matrix (5 g) with methanol/water (4/1, v/v, 100 mL) in the presence of Celite. An aliquot (10 mL) is filtered, concentrated after addition of internal standard (0.20 mL at 0.25 µg/mL) and cleaned-up by simple dispersive solid phase extraction (Bakerbond C18 SPE). Final quantitation is accomplished by liquid chromatography using differential mobility and triple-quadrupole mass spectrometry (LC-DMS/MS/MS).

For T, TA (only for tomato, cucumber, lettuce and carrot leaf), TAA and TLA, separation is achieved using a Thermo Aquasil C18 column (3 x 150 mm, 3 µm) with a mobile phase gradient consisting of 0.5% formic acid in water and methanol, respectively, at a flow rate of 0.6 mL/min. For TA and TLA (only for dry bean seed), separation was achieved using a Thermo Hypercarb column (4.6 x 100 mm, 5 µm) at similar conditions. For T, TA, TAA and TLA the mass transitions  $m/z$  70 → 43,  $m/z$  157 → 70,  $m/z$  128 → 70 and  $m/z$  158 → 70 were used as quantifier. For TA, the mass transition  $m/z$  157 → 88 was monitored additionally as qualifier.

### Analytical conditions

System: Series 1200HPLC, Agilent Technologies  
Column: Thermo Aquasil C18 column (150 mm length, 3 mm i.d., 3 µm particle size) or equivalent, with pre-column  
Mobile phase A: Water 0.5% (v/v) formic acid  
Mobile phase B: Methanol + 0.5 % (v/v) formic acid  
Flow: 0.6 mL/min  
Column temperature: 60°C  
Injection volume: 20 µL

	A	B
0 min	95	5
1 min	95	5
1.30 min	60	40
3 min	60	40
3.01 min	5	95
5 min	5	95
5.51 min	95	5
8.50 min	95	5

### Analytical conditions for confirmation

System: Series 1200HPLC, Agilent Technologies  
Column: Thermo Hypercarb column (100 mm length, 4.6 mm i.d., 5 µm particle size) or equivalent, with pre-column  
Mobile phase A: Water 0.5% (v/v) formic acid  
Mobile phase B: Methanol + 0.5 % (v/v) formic acid  
Flow: 0.6 mL/min  
Column temperature: 60°C  
Injection volume: 20 µL

	A	B
0 min	95	5
3 min	95	5
5 min	60	40
8 min	60	40
8.01 min	5	95
11 min	5	95

System: AB SCIEX QTRAP® 5500 system equipped with DMS SelexION® technology  
Ionisation type: Electrospray ionisation (ESI, TurboIon Spray) 600°C  
Polarity: Positive ion mode  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

The following mass transitions were monitored:

Analyte monitored	Mass transition monitored (m/z)
1,2,4-T	70 → 43*
1,2,4-T IS	75 → 46
TA	157 → 70*
	157 → 88
TA IS	162 → 75
TAA	128 → 70*
TAA IS	133 → 75
TLA	158 → 70*
TLA ISTD	163 → 75

\* Quantitation transition

### Results and discussions

For fortifications at 1.0 mg/kg, all average recoveries were within the 70% - 110% bracket with %RSD values below 20%, as per Guidance Document SANTE/2020/12830 rev.1, referencing and superseding Guidance Document SANCO/3029/99, except for 1,2,4-Triazole in cereal grains and cereal green plants, and Triazole Lactic Acid in tomatoes. For fortifications at 0.01 mg/kg, all average recoveries were within the 60% - 120% bracket with %RSD values below 30%, as per Guidance Document SANTE/2020/12830 rev.1.

**Table A 10: Recovery results from method validation of triazole derivative metabolites using the analytical method**

1,2,4-triazole						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
Tomato	0.01*	5	105	10	102	8
	1.0	5	98	5		
Cucumber	0.01*	3	90	14	95	11
	1.0	3	100	3		
Lettuce	0.01*	3	88	8	95	9
	1.0	3	102	1		
Cereal grain	0.01*	5	115	4	117	5
	1.0	5	118	5		
Cereal straw	0.01*	3	109	17	106	16
	1.0	3	102	19		
Cereal green plant	0.01*	3	109	7	112	6
	1.0	3	116	4		
Whole orange	0.01*	5	100	10	100	7
	1.0	5	100	2		
Oilseed rape seed	0.01*	5	102	7	97	8
	1.0	5	93	6		
Melon peel	0.01*	3	94	12	101	11
	1.0	3	108	7		
Melon whole fruit	0.01*	3	98	2	99	6
	1.0	3	100	9		
Melon pulp	0.01*	3	97	5	104	8
	1.0	3	110	2		
Sweet pepper	0.01*	3	87	11	97	14
	1.0	3	107	9		
Dry bean seed	0.01*	5	104	8	100	9
	1.0	5	96	8		
Carrot leaf	0.01*	3	112	6	105	10
	1.0	3	97	6		
Carrot root	0.01*	3	90	5	94	7
	1.0	3	98	6		

\* Limit of quantification, defined by the lowest validated fortification level

Triazole alanine						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
Tomato	0.01*	5	111	14	111	12
	1.0	5	110	12		
Cucumber	0.01*	3	111	13	110	10
	1.0	3	109	7		
Lettuce	0.01*	3	116	6	111	9
	1.0	3	106	9		
Cereal grain	0.01*	5	91	12	87	11
	1.0	5	84	6		
Cereal straw	0.01*	3	79	19	78	12
	1.0	3	76	1		
Cereal green plant	0.01*	3	108	8	104	7
	1.0	3	100	4		
Whole orange	0.01*	5	90	6	95	7
	1.0	5	100	3		
Sunflower seed	0.01*	5	101	25	96	19
	1.0	5	92	5		
Melon peel	0.01*	3	97	27	97	18
	1.0	3	96	7		
Melon fruit	0.01*	3	101	9	104	7
	1.0	3	107	6		
Melon pulp	0.01*	3	77	29	94	25
	1.0	3	110	7		
Sweet pepper	0.01*	3	104	21	104	15
	1.0	3	104	9		
Dry bean seed	0.01*	5	88	12	85	11
	1.0	5	81	9		
Carrot leaf	0.01*	3	118	10	114	11
	1.0	3	110	14		
Carrot root	0.01*	3	98	9	102	6
	1.0	3	105	1		

\* Limit of quantification, defined by the lowest validated fortification level

Triazole acetic acid						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
Tomato	0.01*	5	90	5	95	7
	1.0	5	101	5		
Cucumber	0.01*	3	100	10	103	7
	1.0	3	105	4		
Lettuce	0.01*	3	105	5	104	4
	1.0	3	104	5		
Cereal grain	0.01*	5	97	9	89	13
	1.0	5	80	5		
Cereal straw	0.01*	3	109	17	100	16
	1.0	3	90	9		
Cereal green plant	0.01*	3	103	7	103	5
	1.0	3	102	5		
Whole orange	0.01*	5	92	3	92	3
	1.0	5	92	3		
Oilseed rape seed	0.01*	5	99	13	97	9
	1.0	5	95	4		
Melon peel	0.01*	3	92	7	94	5
	1.0	3	96	2		
Melon fruit	0.01*	3	97	5	103	8
	1.0	3	110	2		
Melon pulp	0.01*	3	99	3	102	6
	1.0	3	105	7		
Sweet pepper	0.01*	3	106	1	108	3
	1.0	3	110	1		
Dry bean seed	0.01*	5	103	11	88	21
	1.0	5	72	7		
Carrot leaf	0.01*	3	106	11	107	8
	1.0	3	108	3		
Carrot root	0.01*	3	104	9	105	6
	1.0	3	105	4		

\* Limit of quantification, defined by the lowest validated fortification level

Triazole lactic acid						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
Tomato	0.01*	5	92	13	103	14
	1.0	5	114	5		
Cucumber	0.01*	3	100	6	104	6
	1.0	3	108	2		
Lettuce	0.01*	3	108	6	106	6
	1.0	3	104	7		
Cereal grain	0.01*	5	80	3	79	4
	1.0	5	79	5		
Cereal straw	0.01*	3	100	6	93	12
	1.0	3	85	12		

Triazole lactic acid						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
Cereal green plant	0.01 *	3	89	7	94	8
	1.0	3	98	5		
Whole orange	0.01 *	5	95	6	93	6
	1.0	5	92	7		
Oilseed rape seed	0.01 *	5	82	10	90	12
	1.0	5	98	3		
Melon peel	0.01 *	3	105	4	99	7
	1.0	3	93	4		
Melon fruit	0.01 *	3	106	10	107	6
	1.0	3	109	2		
Melon pulp	0.01 *	3	103	10	105	7
	1.0	3	108	4		
Sweet pepper	0.01 *	3	107	9	108	6
	1.0	3	110	1		
Dry bean seed	0.01 *	5	91	6	93	5
	1.0	5	94	5		
Carrot leaf	0.01 *	3	118	6	110	9
	1.0	3	102	4		
Carrot root	0.01 *	3	105	5	106	4
	1.0	3	106	4		

\* Limit of quantification, defined by the lowest validated fortification level

**Table A 11: Characteristics for the analytical method used for validation of triazole derivative metabolite residues in plant matrices**

	Triazole derivative metabolites
Specificity	<p>Only one LC-MS/MS MRM transition per analyte was monitored except for triazolyalalanine (TA).</p> <p>Additional injections for triazole acetic acid (TAA) and triazole lactic acid (TLA) using the Hypercarb column monitoring their MRMs in the positive ion modus.</p> <p>Additional injections for triazole acetic acid (TAA) and triazole lactic acid (TLA) on either column monitoring their respective MRMs in the negative ion modus.</p> <p>Using an additional stationary phase for 1,2,4-triazole (T) such as e.g. a phenyl-ether-type phase like the Phenomenex Luna Synergi Polar-RP.</p> <p>Alternatively, confirmation can be achieved by multiple derivatisation with subsequent SPE clean-up and separate LC-MS/MS injections of two final extracts, one for the analysis of derivatized 1,2,4-triazole (T), the other one for derivatised triazolyalalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA), as described in detail in analytical BCS method 01062/M002.</p> <p>Nevertheless, the LC DMS/MS/MS method is considered highly specific for its use for data-generation.</p> <p>Residues of some of the analytes in untreated blank control samples were frequently present, due to natural occurrence in the environment, and used to correct residues of samples fortified at the LOQ to obtain background corrected recoveries. Representative chromatograms and product ion spectra are provided.</p>
Calibration (type, number of data points)	<p>The linearity of the method was demonstrated using calibration standards prepared in water. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were &gt; 0.99.</p> <p>Representative equations of the calibration line:</p> <p>1,2,4-triazole (C18 column): <math>y = 0.837 x + 0.00158</math> (r = 0.9993) (n= 8)</p> <p>Triazolyalalanine (C18 column): <math>y = 1.95 x - 0.000528</math> (r = 0.9987) (n= 8)</p> <p>Triazole acetic acid (C18 column): <math>y = 1.01 x + 0.0018</math> (r = 0.9991) (n= 8)</p> <p>Triazole Lactic acid (C18 column): <math>y = 1.07 x - 0.00333</math> (r = 0.9997) (n= 8)</p> <p>Triazolyalalanine (Hypercarb column): <math>y = 1.61 x + 0.00488</math> (r = 0.9999) (n= 8)</p> <p>Triazole Lactic acid (Hypercarb column): <math>y = 0.801 x + 0.0073</math> (r = 0.9972) (n= 8)</p>
Calibration range	Linearity was confirmed over the calibration range 1 ng/mL to 600 ng/mL, (corresponding to 0.002 mg/kg to 1.2 mg/kg).
Assessment of matrix effects is presented	No, the internal standard procedure, using stable isotopically labelled internal standards, compensates for matrix effects.
Limit of determination/quantification	<p>The method has a LOQ of 0.01 mg/kg for each analyte in the investigated plant matrices, corresponding to the lowest fortification level.</p> <p>The detection limits (LOD) are estimated to be about 0.002 mg/kg.</p>
Stability of standards and extracts	The stability of the analytes and their internal standards in solution and extracts was not tested specifically. Acceptable recoveries (obtained with fortification and internal standard solutions dosed separately) obtained with calibration solutions (with both the analytes and their internal standards present) sufficiently demonstrate stability.

## Conclusion

This analytical method (01062/M004) has been previously submitted and considered acceptable (data owners: TDMG. Previously submitted in TDM addendum – confirmatory data, UK, 2018). This analytical method for the determination of 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid content in various plant matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/3029/99 rev.4 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for triazole derivative metabolites in plant matrices.

### A 2.1.1.2.1.5 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.1.6 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

#### A 2.1.1.2.2 Analytical method – Determination of prothioconazole in water

##### A 2.1.1.2.2.1 Method validation

Comments of zRMS:	<p>The analytical method MA RES 147-1 has been validated for the determination of prothioconazole in test medium with regards to specificity, linearity, accuracy and precision in accordance with guideline SANCO/3029/99, rev. 4.</p> <p>Specificity for prothioconazole, in treated water, was demonstrated by the absence of significant interferences above 30% of the LOQ.</p> <p>The analytical calibration was shown to be linear (<math>r \geq 0.998</math>) over the range of 50 to 2000 ng prothioconazole/mL.</p> <p>Accuracy was confirmed with recovery of spiked samples at relevant concentrations of the test item in matrix water (10 and 100000 mg prothioconazole/L).</p> <p>The limit of quantification (LOQ) for prothioconazole in treated water was 10 mg/L. The limit of detection (LOD) for prothioconazole in water was 2.5 mg/L.</p> <p>Mean recoveries were in the range of 70 – 110% with relative standard deviations of <math>\leq 20\%</math> for prothioconazole at each level.</p> <p>The study is acceptable.</p>
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Reference: KCP 5.1.2/05

Report Analytical Method for the Determination of the content of Prothioconazole in water by UHPLC-TOF-MS/MS according to guideline SANCO/3029/99 rev.4, Morsiani S., 2020, Test Site Code: 20070-01R (Study Code 029SRFR20C05), Analytical Method: MA RES 147-1

Guideline(s): SANCO/3029/99 rev.4

Deviations: No

GLP: Yes

Acceptability: Yes

#### Materials and methods

Homogenised water samples (0.5 g) were extracted with acetonitrile (25 mL) prior to further dilution with acetonitrile into the calibration range, if necessary. Samples were then filtered (PTFE, 0.2  $\mu$ m) and analysed by UHPLC-TOF-MS/MS (two mass transitions monitored, 344.0/326.03 and 344.0/125.02 m/z). Solvent calibration standards prepared in acetonitrile were used for quantitation.

#### Analytical conditions

System: UHPLC Series 1290 Infinity, Agilent  
Column: Zorbax Eclipse plus C18, 2.1 \* 50 mm, 1.8  $\mu$ m particle size  
Mobile phase A: Water 0.1% (v/v) formic acid  
Mobile phase B: Methanol + 0.1 % (v/v) formic acid  
Flow: 0.5 mL/min  
Column temperature: 35°C  
Injection volume: 1  $\mu$ L

	A	B
0 min	80	20
0.5 min	80	20
4 min	5	95
5.5 min	5	95
6 min	80	20
8 min	80	20

System: Triple TOF 4600 AB SCIEX  
Ionisation type: Electrospray ionisation (ESI) 450°C  
Polarity: Positive ion mode  
Ion spray voltage: 5500 V  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
MRM monitored for prothioconazole (1): 326.0266-326.0298 (quantifier)  
MRM monitored for prothioconazole (2): 125.0146-125.0158 (qualifier)

## Results and discussions

**Table A 12: Recovery results from method validation of prothioconazole using the analytical method**

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Water	Prothioconazole	10	99.4	2.5	quantifier
		100 000	92.1	8.6	quantifier
		Overall	95.7	7.1	quantifier
		10	100.2	2.2	qualifier
		100 000	92.1	7.9	qualifier
		Overall	96.2	6.9	qualifier

**Table A 13: Characteristics for the analytical method used for validation of prothioconazole residues in water**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two product ions of the parent ion. TOF-MS/MS is a very highly specific analytical technique. The untreated control samples showed no significant interference above 30 % of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Representative calibration equation: Quantifier: $y = 35.88136x - 915.72710$ (n = 6) $r = 0.99811$ Qualifier: $y = 3.83712x - 87.86390$ (n = 6) $r = 0.99859$
Calibration range	50.30 to 2012 ng/mL equivalent to 2.5 to 100 mg/kg for undiluted samples (n=6).
Assessment of matrix effects is presented	Yes (not significant)
Limit of determination/quantification	LOQ for prothioconazole in water = 10 mg/kg. LOD for prothioconazole in water = 2.5 mg/kg, determined (lowest detectable concentration, prepared for the prothioconazole calibration in matrix-matched standards).
Stability of standards and extracts	Not assessed in this study.

## Conclusion

The method validation is considered valid and acceptable according SANCO/3029/99 rev. 4 for the determination of prothioconazole in water at an LOQ of 10 mg/kg (10 mg/L). Validation also complies with SANTE/2020/12830, Rev.1.

### A 2.1.1.2.2.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

#### A 2.1.1.2.2.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

#### A 2.1.1.2.3 Analytical method – Determination of prothioconazole in sucrose solutions

##### A 2.1.1.2.3.1 Method validation

Comments of zRMS:	<p>The analytical method MA RES 148-1 has been validated for the determination of prothioconazole in sucrose feeding solutions of honey bees with regards to specificity, linearity, accuracy and precision in accordance with guideline SANCO/3029/99, rev. 4.</p> <p>Specificity for prothioconazole, in sucrose feeding solutions, was demonstrated by the absence of significant interferences above 30% of the LOQ.</p> <p>The analytical calibration was shown to be linear (<math>r \geq 0.995</math>) over the range of 0.005 to 0.25 mg/kg in undiluted samples (the first linearity range) and from 0.15 to 5 mg/kg in undiluted samples (the second range).</p> <p>Accuracy was confirmed with recovery of spiked samples at relevant concentrations of the test item in feeding solution (0.018 and 6000 mg prothioconazole/kg).</p> <p>The limit of quantification (LOQ) for prothioconazole in sucrose feeding solution was 0.018 mg/kg (corresponding to 0.022 mg/L, considering the density of the sucrose solution at 20°C, 1.219 g/mL).</p> <p>Mean recoveries were in the range of 70 – 110% with relative standard deviations of <math>\leq 20\%</math> for prothioconazole at each level.</p> <p>The study is acceptable.</p>
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Reference: KCP 5.1.2/06

Report Analytical Method for the Determination of the content of Prothioconazole in sucrose feeding solutions of honey bees by UHPLC-TOF-MS/MS according to guideline SANCO/3029/99 rev.4, Morsiani S., 2020, Test Site Code: 20070-02R (Study Code 029SRFR20C04), Analytical Method: MA RES 148-1

Guideline(s): SANCO/3029/99 rev.4

Deviations: No

GLP: Yes

Acceptability: Yes

#### Materials and methods

Homogenised sucrose solution samples (0.2 g) were extracted with a combination of acetonitrile (2 mL) and water (up to 10 mL) prior to further dilution with blank sample into the calibration range, if necessary. Samples were then filtered (MCE, 0.2  $\mu$ m) and analysed by UHPLC-TOF-MS/MS (two mass transitions monitored, 344.0/326.03 and 344.0/125.02 m/z). Matrix-matched calibration standards were used for quantitation.

#### Analytical conditions

System: UHPLC Series 1290 Infinity, Agilent  
Column: Zorbax Eclipse plus C18, 2.1 \* 50 mm, 1.8  $\mu$ m particle size  
Mobile phase A: Water 0.1% (v/v) formic acid

Mobile phase B: Methanol + 0.1 % (v/v) formic acid  
Flow: 0.5 mL/min  
Column temperature: 35°C

Injection volume: 20 µL or 1 µL

	A	B
0 min	80	20
0.5 min	80	20

4 min	5	95
5.5 min	5	95
6 min	80	20
8 min	80	20

System: Triple TOF 4600 AB SCIEX  
Ionisation type: Electrospray ionisation (ESI) 450°C  
Polarity: Positive ion mode  
Ion spray voltage: 5500 V  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
MRM monitored for prothioconazole (1): 326.0266-326.0298 (quantifier)  
MRM monitored for prothioconazole (2): 125.0146-125.0158 (qualifier)

## Results and discussions

**Table A 14: Recovery results from method validation of prothioconazole using the analytical method**

Matrix	Analyte	Fortification level (mg/kg) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Sucrose solution	Prothioconazole	0.018	92.8	5.0	quantifier
		6000	95.5	11.3	quantifier
		Overall	94.2	8.5	quantifier
		0.018	100.8	7.7	qualifier
		6000	94.2	11.9	qualifier
		Overall	97.5	10.0	qualifier

**Table A 15: Characteristics for the analytical method used for validation of prothioconazole residues in sucrose solution**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two product ions of the parent ion. TOF-MS/MS is a very highly specific analytical technique. Control samples showed no significant interference above 30% of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Representative calibration equations: Quantifier: 1.011 to 50.56 ng/mL: $y = 1692.14424x - 579.16673$ (n = 6) $r = 0.99811$ 30.34 to 1011 ng/mL: $y = 84.54430x + 44.38314$ (n = 6) $r = 0.99703$ Qualifier: 1.011 to 50.56 ng/mL: $y = 168.01194x - 28.70609$ (n = 6) $r = 0.99563$ 30.34 to 1011 ng/mL: $y = 8.43730 + 20.88223$ (n = 6) $r = 0.99843$
Calibration range	Two linearity ranges were established from 1.011 to 50.56 ng/mL (equivalent to 0.005 to 0.25 mg/kg in undiluted samples) and from 30.34 to 1011 ng/mL (equivalent to 0.15 to 5 mg/kg in undiluted samples).
Assessment of matrix effects is presented	Yes (significant for qualifier ion)
Limit of determination/quantification	LOQ for prothioconazole in sucrose feeding solutions = 0.018 mg/kg (corresponding to 0.022 mg/L, considering the density of the sucrose solution)

	Prothioconazole
	at 20°C, 1.219 g/mL). LOD for prothioconazole in sucrose feeding solutions = 0.005 mg/kg, (lowest detectable concentration, prepared for the prothioconazole calibration in matrix-matched standards).
Stability of standards and extracts	Not assessed in this study.

## Conclusion

The method validation is considered valid and acceptable according SANCO/3029/99 rev. 4 for the determination of prothioconazole in sucrose solutions at an LOQ of 0.018 mg/kg. Validation also complies with SANTE/2020/12830, Rev.1.

### A 2.1.1.2.3.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.3.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### A 2.1.1.2.4 Analytical method – Determination of prothioconazole in test medium (to support Lemna studies)

#### A 2.1.1.2.4.1 Method validation

Comments of zRMS:	The method of analysis has been validated for the determination of prothioconazole in test medium according to SANCO/3029/99 rev. 4 to meet the requirements for specificity, linearity, accuracy, precision-repeatability, limit of quantification (LOQ). The LOQ is 0.257 µg active ingredient/L, corresponding to 0.00100 mg test item/L. The acceptance criteria of the guideline (accuracy within 70% to 110% and precision <20%) were met. The method is acceptable.
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Reference: KCP 5.1.2/07

Report 250 EC Prothioconazole (CA3301) – Effect on the Aquatic Higher Plant *Lemna gibba* in a 7-Day Growth Inhibition Test, Semal S. (Study Director Dupont A.), 2021, 20190456

Guideline(s): SANCO/3029/99 rev.4

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The test water samples (10 mL), completely diluted with 10 mL of acetonitrile prior to storage, were thawed at room temperature for 1 hour. If necessary, the samples were further diluted into the linear range with acetonitrile / test water (1/1, v/v). Samples were analysed by HPLC-MS/MS (two mass transitions

monitored, 344/189 and 344/125 m/z). Matrix-matched calibration standards were used for quantitation.

### Analytical conditions

System: HPLC Series 1290 Infinity II, Agilent  
Column: Waters Acquity UPLC C18, 50 × 2.1 mm; 1.7 µm  
Mobile phase A: Water + 0.1% (v/v) formic acid  
Mobile phase B: Acetonitrile + 0.1 % (v/v) formic acid  
Flow: 0.5 mL/min  
Column temperature: 40°C  
Injection volume: 1 µL

	A	B
0 min	70	30
2 min	10	90
2.9 min	10	90
3.0 min	70	30
4.0 min	70	30

System: AB Sciex/QTRAP 6500+  
Ionisation type: Electrospray ionisation (ESI)  
Polarity: Positive ion mode  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
MRM monitored for prothioconazole: 344-189 m/z (quantifier)  
MRM monitored for prothioconazole: 344-125 m/z (qualifier)

### Results and discussions

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

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Test medium	Prothioconazole	0.001 mg test item/L 0.257 µg prothioconazole/L	109	8.0	quantifier
		3.03 mg test item/L 775 µg prothioconazole/L	106	2.4	quantifier
		0.001 mg test item/L 0.257 µg prothioconazole/L	109	5.9	qualifier
		3.03 mg test item/L 775 µg prothioconazole/L	105	2.1	qualifier

**Table A 17: Characteristics for the analytical method used for validation of prothioconazole residues in test medium**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two mass transitions. Control samples (analytical blank and biological control samples) showed no significant interference above 30 % of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Quantifier: Representative calibration equation: $y = 110164x^{1.0144}$ (n = 8) $R^2 = 0.9998$ Qualifier: Representative calibration equation: $y = 65\,439x^{1.0267}$ (n = 8) $R^2 = 0.9998$
Calibration range	0.0490 – 48.6 µg prothioconazole/L (corresponding to 0.192 to 190 µg test item/L)
Assessment of matrix effects is presented	No
Limit of determination/quantification	The limit of quantification (LOQ) for prothioconazole in the test samples was derived from the lowest concentration of spiked samples which was validated. The LOQ is 0.257 µg prothioconazole/L, corresponding to 0.00100 mg test

	Prothioconazole
	item/L. The limit of detection (LOD) for prothioconazole in the test samples was derived from the lowest calibration solution. Taking into account a sample preparation factor of 2, the LOD is 0.0980 µg prothioconazole/L.
Stability of standards and extracts	Not assessed in this study.

## Conclusion

The method validation is considered valid and acceptable according SANCO/3029/99 rev. 4 for the determination of prothioconazole in test medium at an LOQ of 0.257 µg prothioconazole/L, corresponding to 0.00100 mg test item/L. Validation also complies with SANTE/2020/12830, Rev.1.

### A 2.1.1.2.4.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.4.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### A 2.1.1.2.5 Analytical method – Determination of prothioconazole in test medium (to support *Skeletonema* studies)

#### A 2.1.1.2.5.1 Method validation

Comments of zRMS:	The method of analysis has been validated for the determination of prothioconazole in test medium according to SANCO/3029/99 rev. 4 to meet the requirements for specificity, linearity, accuracy, precision-repeatability, limit of quantification (LOQ). The LOQ is 0.389 µg prothioconazole/L, corresponding to 0.00152 mg test item/L. The acceptance criteria of the guideline (accuracy within 70% to 110% and precision <20%) were met. The method is acceptable.
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Reference: KCP 5.1.2/08

Report 250 EC Prothioconazole (CA3301) - Effect on *Skeletonema sp.* in a 72-Hour Algal Growth Inhibition Test, Semal S. (Study Director Dupont A.), 2021, 20190454

Guideline(s): SANCO/3029/99 rev.4

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The water test samples (10 mL), completely diluted with 10 mL of acetonitrile prior to storage, were thawed at room temperature for 1 hour. The test and control samples from day 1, 2 and 3 were centrifuged due to the presence of algae in addition to an aliquot from one sample of each spike level. If necessary, the samples were further diluted into the linear range with acetonitrile / test water (1/1, v/v). Samples were analysed by HPLC-MS/MS (two mass transitions monitored, 344/189 and 344/125 m/z). Matrix-

matched calibration standards were used for quantitation.

### Analytical conditions

System: HPLC Series 1290 Infinity II, Agilent  
Column: Waters Acquity UPLC C18, 50 × 2.1 mm; 1.7 µm  
Mobile phase A: Water + 0.1% (v/v) formic acid  
Mobile phase B: Acetonitrile + 0.1 % (v/v) formic acid  
Flow: 0.5 mL/min  
Column temperature: 40°C  
Injection volume: 1 µL

	A	B
0 min	70	30
2 min	10	90
2.9 min	10	90
3.0 min	70	30
3.5 min	70	30

System: AB Sciex/QTRAP 6500+  
Ionisation type: Electrospray ionisation (ESI)  
Polarity: Positive ion mode  
Scan type: MS/MS, Multiple Reaction Monitoring (MRM)  
MRM monitored for prothioconazole: 344-189 m/z (quantifier)  
MRM monitored for prothioconazole: 344-125 m/z (qualifier)

### Results and discussions

**Table A 18: Recovery results from method validation of prothioconazole using the analytical method**

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Test medium	Prothioconazole	0.00152 mg test item/L 0.389 µg prothioconazole/L	87	12.5	quantifier
		1.00 mg test item/L 256 µg prothioconazole/L	99	4.2	quantifier
		0.00152 mg test item/L 0.389 µg prothioconazole/L	85	17.1	qualifier
		1.00 mg test item/L 256 µg prothioconazole/L	105	5.8	qualifier

**Table A 19: Characteristics for the analytical method used for validation of prothioconazole residues in test medium**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two mass transitions. Control samples (analytical blank and biological control samples) showed no significant interference above 30 % of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Quantifier: Representative calibration equation: $y = 19907x^{1.0525}$ (n = 9) $R^2 = 0.9993$ Qualifier: Representative calibration equation: $y = 14784 x^{1.0284}$ (n = 9) $R^2 = 0.9991$
Calibration range	0.0474 – 23.7 µg prothioconazole/L (corresponding to 0.185 to 92.7 µg test item/L)
Assessment of matrix effects is presented	No
Limit of determination/quantification	The limit of quantification (LOQ) for prothioconazole in the test samples was derived from the lowest concentration of spiked samples which was validated. The LOQ is 0.389 µg prothioconazole/L, corresponding to 0.00152 mg test item/L.

	Prothioconazole
	The limit of detection (LOD) for prothioconazole in the test samples was derived from the lowest calibration solution. Taking into account a sample preparation factor of 2, the LOD is 0.0948 µg prothioconazole/L.
Stability of standards and extracts	Not assessed in this study.

## Conclusion

The method validation is considered valid and acceptable according SANCO/3029/99 rev. 4 for the determination of prothioconazole in test medium at an LOQ of 0.389 µg prothioconazole/L, corresponding to 0.00152 mg test item/L. Validation also complies with SANTE/2020/12830, Rev.1.

### A 2.1.1.2.5.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.5.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### A 2.1.1.2.6 Analytical method – Determination of prothioconazole in water (to support Honey Bee Larval Toxicity studies)

#### A 2.1.1.2.6.1 Method validation

Comments of zRMS:	The method of analysis has been validated for the determination of prothioconazole in deionised water according to SANTE/2020/12830 Rev.1 to meet the requirements for specificity, linearity, accuracy, precision-repeatability, limit of quantification (LOQ). The limit of quantification (LOQ) was 0.7 mg prothioconazole/L in deionised water. The mean recovery at each fortification level was in the range of 70 - 120% with a relative standard deviation of ≤20% and thus comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, Rev.1. The method is acceptable.
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Reference: KCP 5.1.2/09

Report CA3301 (Prothioconazole 250 EC, NUL 3390): Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under Laboratory Conditions, Ortiz M. G., 2021, S20-09403

Guideline(s): SANTE/2020/12830, Rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

Deionised water samples (2 mL) were fortified, if necessary, and diluted with acetonitrile/water (1/1, v/v) + 10 % of 250 g/L L-cysteine solution (to 40 mL). The samples were further diluted with acetonitrile/water (1/1, v/v) + 10 % of 250 g/L L-cysteine solution (5 fold). If necessary, the samples were further diluted with matrix blank extract to be within the calibration range. Samples were analysed by

HPLC-MS/MS (two mass transitions monitored, 344/189 and 344/154 m/z). Matrix-matched calibration standards were used for quantitation.

### Analytical conditions

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  $\mu$ m Biphenyl 100A, 100 mm x 2.1 mm, 2.6  $\mu$ m

Mobile phase A: Water + 0.1% (v/v) acetic acid

Mobile phase B: Acetonitrile + 0.1 % (v/v) acetic acid

Flow: 0.6 mL/min

Column temperature: 40°C

Injection volume: 5  $\mu$ L

	A	B
0 min	70	30
1 min	70	30
3.5 min	1	99
4.5 min	1	99
4.6 min	70	30
6.0 min	70	30

System: SCIEX API 5500

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

MRM monitored for prothioconazole: 344-189 m/z (quantifier)

MRM monitored for prothioconazole: 344-154 m/z (qualifier)

### Results and discussions

**Table A 20: Recovery results from method validation of prothioconazole using the analytical method**

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)	Comments
Test medium	Prothioconazole	0.7	100	2	Quantifier
		1000	102	2	Quantifier

**Table A 21: Characteristics for the analytical method used for validation of prothioconazole residues in deionised water**

	Prothioconazole
Specificity	MS/MS determination was conducted by monitoring two mass transitions. Control samples showed no significant interference above 30% of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms and product ion spectrum are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Representative calibration equation: $y = 6.56e+004 x + 5.52e+003$ (n = 6) $r = 0.9998$
Calibration range	2.10 – 38.0 ng/mL (corresponding to 0.21 to 3.8 mg/L)
Assessment of matrix effects is presented	Yes (not significant)
Limit of determination/quantification	LOQ = 0.7 mg prothioconazole/L LOD = 0.21 mg prothioconazole/L (lowest calibration standard)
Stability of standards and extracts	The stability of stock solution of prothioconazole in acetonitrile was demonstrated in EAS Study No. S20-09747 for 14 days. The stock solution (78660 mg/L prothioconazole in acetonitrile) and its dilution (100 mg/L in water) were freshly prepared in demineralised water and used for fortification within 24 hours after preparation. Therefore, investigation of the stability of this fortification solution was not necessary in the present study. Extract stability is not considered to be an issue since matrix-matched standards that were used for quantification were always prepared on the same day as the

	Prothioconazole
	work up of the sample for residue analysis took place. Furthermore, the interval from preparation of the final extracts to injection did not exceed 24 hours.

## Conclusion

The method validation is considered valid and acceptable according SANTE/2020/12830, Rev. 1 for the determination of prothioconazole in deionised water at an LOQ of 0.7 mg prothioconazole/L.

### A 2.1.1.2.6.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

### A 2.1.1.2.6.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

### A 2.1.1.2.7 Analytical method – Determination of prothioconazole in acetonitrile rinse and tank mix solutions (Effectiveness of cleaning study)

#### A 2.1.1.2.7.1 Method validation

Comments of zRMS:	The method is sufficiently described and is considered fit for purpose.
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Reference:	KCP 5.1.2/10
Report	CA3301 – Effectiveness of Cleaning, Calvert A., 2022, 22/1499
Guideline(s):	CRD Efficacy Guideline 305
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Materials and methods

Tank mix solutions: 1 ml aliquots of the tank mix were taken into two separate 100 ml volumetric flasks and made to volume with acetonitrile. Each solution was analysed singly.

Acetonitrile rinse solutions: Samples of the acetonitrile rinse were analysed directly without further dilution. If necessary, the samples were further diluted to be within the calibration range.

Samples were analysed by HPLC-UV. Solvent calibration standards were used for quantitation.

## Analytical conditions

System: HPLC with UV detection  
Column: Phenomenex Kinetex, 2.6 µm, C18, 100 x 4.6 mm  
Mobile phase: 50/50 v/v, Acetonitrile: 0.1% v/v Formic Acid  
Flow rate: 1.0 mL/min

Column temperature: 40°C  
Injection volume: 10 µL  
Detector Wavelength: 254 nm

## Results and discussions

**Table A 22: Recovery results from method validation of prothioconazole using the analytical method**

Matrix	Analyte	Fortification level (mg/L) (n = 5)	Mean recovery (%)	RSD (%)
Acetonitrile	Prothioconazole	0.5	107.8	4.0
		400	100.5	0.2

**Table A 23: Characteristics for the analytical method used for validation of prothioconazole residues in acetonitrile rinse and tank mix solutions**

	Prothioconazole
Specificity	Blank solutions of tap water, hardwater D, acetonitrile and 1.5 mg/ml solution of formulation blank in acetonitrile samples showed no significant interference above 30 % of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Representative chromatograms are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using calibration standards in acetonitrile. Linear calibration functions were calculated by regression analysis. The correlation coefficients, r obtained were > 0.99. Representative calibration equation: $y = 342.190799 x$ (n=5) $r = 0.9998$
Calibration range	0.0004 – 0.5 mg/mL (corresponding to 0.4 to 500 mg/L)
Assessment of matrix effects is presented	Not relevant, as the matrix is the solvent used for the calibration.
Limit of determination/quantification	LOQ = 0.5 mg prothioconazole/L LOD = 0.4 mg prothioconazole/L (lowest calibration standard)
Stability of standards and extracts	The stock solution and its dilution, and the extracts were freshly prepared and used within 24 hours after preparation. Therefore, investigation of the stability of standards and extracts was not necessary in the present study.

## Conclusion

The method validation is considered valid and acceptable according CRD Efficacy Guideline 305 for the determination of prothioconazole in acetonitrile rinse and tank mix solutions at an LOQ of 0.5 mg prothioconazole/L.

### A 2.1.1.2.7.2 Confirmatory method (if required)

Confirmatory method is not required for methods for risk assessment according to SANTE/2020/12830, Rev.1.

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

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

No new or additional studies have been submitted.

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

##### A 2.1.2.2.1 Analytical method 1 – prothioconazole and metabolites in pollinator matrices

##### A 2.1.2.2.1.1 Method validation

Comments of zRMS:	<p>The analytical method has been successfully validated according to the guidance document SANTE/2020/12830, rev. 1 for the determination of prothioconazole, prothioconazole-desthio (Group 1) and 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid (Group 3) with the tested LOQ of 0.01 mg/kg and 0.01 mg/kg prothioconazole-desthio equivalent for prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio (Group 2) in pollen and nectar. The method was found to be valid at the same levels in honey for prothioconazole-desthio and group 2 and 3.</p> <p>For sugar solution the lowest level was validated for 6.65 mg/kg prothioconazole.</p> <p>All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions for honey and one (1) for all other matrices were within 70 – 120 % with relative standard deviations <math>\leq 20</math> % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1.</p> <p>The method is acceptable.</p>
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Reference: KCP 5.2/01

Report Amendment 1 to Final Report and Final Report  
Development and Validation of Analytical Methods for the Determination of Prothioconazole in different Matrices, Kalathoor R., 2021, Report No.S20-09747

Guideline(s): SANTE/2020/12830, rev. 1

Deviations: No

GLP: Yes

Acceptability: yes

## Materials and methods

In this study, a validation was conducted for prothioconazole, prothioconazole-desthio (Group1), prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio (Group 2), as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid (Group 3) in pollen and nectar for risk assessment. Group 1 (without prothioconazole), Group 2 and Group 3 was validated in honey for post-approval and monitoring purposes. Furthermore, prothioconazole was validated in sugar solution for risk assessment.

### Group 1

#### Prothioconazole / prothioconazole-desthio

Honey (1 g) extracted with a mixture of water (10 mL) and acetonitrile (10 mL)

A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken to obtain phase separation after centrifugation.

Extracts were diluted with methanol/water 40/60 v/v containing 50 g/L cysteine hydrochloride (0.2 mL to 1 mL) for stabilisation of prothioconazole.

Pollen samples (0.2 g) extracted with 6 mL acetonitrile/water 1/1 v/v mixture containing and 0.6 mL 250 g/L cysteine hydrochloride solution.

Lysing Matrix D and a salt mixture of magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken to obtain phase separation by centrifugation. An aliquot (1.2 mL) of the acetonitrile phase was cleaned by adding primary secondary amine (PSA).

Extracts were diluted with methanol/water 40/60 v/v containing 50 g/L cysteine hydrochloride (0.5 mL to 1

mL) for stabilisation of prothioconazole.

Nectar samples (0.2 g) were extracted with 5 mL acetonitrile/water 1/1 v/v and 0.5 mL 250 g/L cysteine hydrochloride solution completed to 10 mL with methanol/water 40/60 v/v and diluted with methanol/water 40/60 v/v (0.5 mL to 1 mL).

## Group 2

**prothioconazole-alpha-hydroxy-desthio,  
prothioconazole-3-hydroxy-desthio,  
prothioconazole-4-hydroxy-desthio,  
prothioconazole-5-hydroxy-desthio and  
prothioconazole -6-hydroxy-desthio**

Honey (1 g) extracted with a mixture of water (10 mL) and acetonitrile (10 mL)

A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken to obtain phase separation by centrifugation.

Extracts were diluted with 10 mM ammonium formate (0.25 mL to 1 mL).

Pollen samples (0.2 g) extracted with 6 mL acetonitrile/water 1/1 v/v mixture containing and 0.6 mL 250 g/L cysteine hydrochloride solution.

Lysing Matrix D and a salt mixture of magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken to obtain phase separation after centrifugation. An aliquot (1.0 mL) of the acetonitrile phase was cleaned by adding primary secondary amine (PSA).

Extracts were diluted with 10 mM ammonium formate (0.5 mL to 1 mL).

Nectar samples (0.1 g) were extracted with 2 mL acetonitrile/water 1/1 v/v and diluted with 10 mM ammonium formate (0.5 mL to 1 mL).

Fortification was done for recovery samples.

Sugar solution (1.19 g = 1 mL) for quantification of prothioconazole was diluted with acetonitrile/water

## Group 3

**1,2,4-triazole, triazole  
alanine, triazole acetic acid  
and triazole lactic acid**

Honey (1 g) extracted with water (4 mL) and the extract was filtered and diluted with HPLC water (0.2 mL to 1 mL).

Pollen samples (0.2 g) extracted with 2x2 mL methanol/water 1/1 v/v. After evaporation of methanol, completion to 4 mL water and addition of C18 powder, extracts were filtered and diluted with HPLC water (0.6 mL to 1 mL).

Nectar samples (0.2 g) extracted with water (8 mL).

containing 10% cysteine hydrochloride (40 mL) and further diluted with same solvent to fit into the calibration range (0.02 mL to 1 mL).

Quantification was performed by use of LC-MS/MS (group 1 and 2) and LC-DMS-MS/MS (group 3) detection using matrix matched calibration.

**Analytical conditions group 1 (Prothioconazole / prothioconazole-desthio)**

System: 1290 Infinity II Binary LC System, Agilent Technologies

Pre-column: HPLC guard column (KJO-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)

Column: Agilent Zorbax Eclipse XDB-C18, 50 mm x 2.1 mm, 3.5 µm

Mobile phase A: Water + 0.1% (v/v) formic acid

Mobile phase B: Acetonitrile + 0.1 % (v/v) formic acid

Flow: 0.8 mL/min

Column temperature: 40°C

Injection volume: 40 µL

	A	B
0 min	80	20
2 min	80	20
5 min	10	90
6.5 min	10	90
7.5 min	80	20
8.5 min	80	20

System: Sciex API 6500+ Linear Ion Trap Quadrupole LC/MS/MS spectrometer

Ionisation type: Electrospray ionisation (ESI, TurboIonSpray)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage: 5000 V

**Analytical conditions Prothioconazole in sugar solutions**

System: Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP

Pre-column: HPLC guard column (KJO-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)

Column: Phenomenex Kinetex 2.6 µm Biphenyl 100A, 100 mm x 2.1 mm, 2.6 µm

Mobile phase A: Water + 0.1% (v/v) acetic acid

Mobile phase B: Acetonitrile + 0.1 % (v/v) acetic acid

Flow: 0.6 mL/min

Column temperature: 40°C

Injection volume: 5 µL

	A	B
0 min	70	30
1 min	70	30
3.5 min	1	99
4.5 min	1	99
4.6 min	70	30
6.0 min	70	30

System: SCIEX API 5500

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage: 3000 V

**Analytical conditions group 2 (prothioconazole-alpha-hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole -6-hydroxy-desthio)**

System: Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP / 1290 Infinity II Binary LC System, Agilent Technologies

Pre-column: UHPLC guard column (AJ0-9000, Phenomenex) with 2.1 mm C18 cartridge (AJ0-8782, Phenomenex)

Column: Agilent Poroshell 120 Phenyl Hexyl (100 mm x 3 mm, 2.7 µm,

Mobile phase A: Water + 5 mM Ammonium formate + 0.1% formic acid

Mobile phase B: Methanol + 5 mM Ammonium formate

+ 0.1% formic acid

Flow: 0.8 mL/min

Column temperature: 60°C

Injection volume: 20 µL

	A	B
0 min	70	30
0.15 min	70	30
0.16 min	50	50
2.6 min	22	78
3.5 min	20	80
4.0 min	10	90

5.0 min	10	90
5.01 min	70	30

6.0 min	70	30
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Same for pollen method validation except:

Mobile phase A: Water + 10 mM Ammonium formate + 0.1% formic acid

Mobile phase B: Methanol + 10 mM Ammonium formate + 0.1% formic acid

Flow: 0.8 mL/min

Column temperature: 60°C

Injection volume: 25 µL

	A	B
0 min	70	30
0.10 min	70	30
0.11 min	50	50
3.0 min	10	90
4.0 min	10	90
4.01 min	70	30
5.5 min	70	30

System: Sciex API QTRAP 5500 Linear Ion Trap Quadrupole LC/MS/MS spectrometer

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage: 5500 V

### Analytical conditions group 3 (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid)

System: 1290 Infinity II HPLC System, Agilent Technologies

Injection volume: 40 µL

Pre-column: HPLC guard column (KJO-4282, Phenomenex) with 4 mm Fusion RP cartridge (AJ0-7556, Phenomenex)

Column: Thermo Hypercarb (100 mm x 3 mm, 5 µm,

Mobile phase A: Water + 0.5% (v/v) formic acid

Mobile phase B: Acetonitrile + 0.5% (v/v) formic acid

Flow: 0.6 mL/min

Column temperature: 30°C

	A	B
0 min	99	1
0.2 min	99	1
5 min	10	90
7 min	10	90
7.01 min	99	1
8.5 min	99	1

Same 1,2,4-triazole (confirmation honey), triazole acetic acid and triazole lactic acid (quantification pollen) except:

Pre-column: HPLC guard column (KJO-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)

Column: Phenomenex Kinetex 2.6 µm Biphenyl 100 (100 mm x 4.6 mm, 2.6 µm

Mobile phase A: Water + 0.5% formic acid

Mobile phase B: Methanol + 0.5% formic acid

Column temperature: 30°C

Injection volume: 40 µL (honey) – 20 µL (pollen)

	A	B	Flow (µL/min)
0 min	95	5	525
5 min	5	95	525
6 min	5	95	700
6.01 min	95	5	700
7.5 min	95	5	700

System: SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer with device for ion mobility spectrometry (SelexION))

Ionisation type: Electrospray ionisation (ESI, TurboIonSpray)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage: 5500 V

Two mass transitions were monitored for each analyte (with the exception of prothioconazole). The mass transitions are detailed below:

Analyte monitored	Mass transition monitored (m/z)	Matrix
Prothioconazole (PTZ)	344 → 154#	Honey, pollen, nectar
	346 → 227	
	344 → 189#	Sugar solution
	344 → 154	
PTZ-desthio	312→125#	Honey, pollen, nectar
	312→70	
PTZ-alpha-hydroxy-desthio	328 → 70#	
	328 → 141	
PTZ-3-hydroxy-desthio	328 → 70#	
	328 → 141	
PTZ-4-hydroxy-desthio	328 → 70#	
	328 → 141	
PTZ-5-hydroxy-desthio	328 → 70#	
	328 → 141	
PTZ-6-hydroxy-desthio	328 → 70#	
	328 → 141	
1,2,4-Triazole	70 → 43#	
	70 → 70	
Triazole Alanine (TA)	157 → 70#	
	157 → 88	
Triazole Acetic Acid (TAA)	128 → 70#	
	128 → 43	
Triazole Lactic Acid (TLA)	158 → 70#	
	158 → 43	

# proposed (and used) for quantification but all of the mass transitions listed can be used for quantification

## Results and discussions

**Table A 24: Recovery results from method validation of prothioconazole and its metabolites using the analytical method**

Analyte	Matrix	Fortification Level (mg/kg) (n = 5)	Mean Recovery (%)	Rel. Std. Dev. (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments
Prothioconazole	Pollen	0.01	107	2	109	2	344→154 m/z (Quantification)
		0.1	110	2			
	Nectar	0.01	96	2	98	2	
		0.1	99	2			
	Sugar solution	6.65	102	2	102	1	344→189 m/z (Quantification)
		6390	103	1			
		8710	102	1			
Prothioconazole-desthio	Pollen	0.01	108	3	109	3	312→125 m/z (Quantification)
		0.1	111	2			
	Nectar	0.01	103	2	102	2	
		0.1	100	1			
	Honey	0.01	100	2	100	1	
		0.1	101	1			
		0.01	100	1	100	1	312→70 m/z (Confirmation)
		0.1	100	1			
Prothioconazole-alpha-hydroxy-Desthio*	Pollen	0.01	109	4	107	4	328→70 m/z (Quantification)
		0.1	105	2			
	Nectar	0.01	95	2	97	2	
		0.1	98	1			
	Honey	0.01	103	3	101	3	
		0.1	100	2			
		0.01	105	4	103	4	328→141 m/z (Confirmation)
		0.1	101	2			
Prothioconazole-3-hydroxy-Desthio*	Pollen	0.01	100	7	102	5	328→70 m/z (Quantification)
		0.1	103	2			
	Nectar	0.01	100	1	99	1	
		0.1	99	1			

	Honey	0.01	103	1	102	2	328→141 <i>m/z</i> (Confirmation)	
		0.1	100	2				
		0.01	101	3	101	3		328→70 <i>m/z</i> (Quantification)
		0.1	100	2				
Prothioconazole-4-hydroxy-Desthio*	Pollen	0.01	105	4	105	4	328→70 <i>m/z</i> (Quantification)	
		0.1	104	4				
	Nectar	0.01	96	2	96	1		328→141 <i>m/z</i> (Confirmation)
		0.1	96	1				
	Honey	0.01	103	1	102	2	328→141 <i>m/z</i> (Confirmation)	
		0.1	100	2				
		0.01	102	2	101	2		328→141 <i>m/z</i> (Confirmation)
		0.1	100	1				
Analyte	Matrix	Fortification Level (mg/kg) (n = 5)	Mean Recovery (%)	Rel. Std. Dev. (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments	
Prothioconazole-5-hydroxy-Desthio*	Pollen	0.01	93	3	98	6	328→70 <i>m/z</i> (Quantification)	
		0.1	102	3				
	Nectar	0.01	99	3	99	2		328→141 <i>m/z</i> (Confirmation)
		0.1	98	1				
	Honey	0.01	103	5	101	4	328→141 <i>m/z</i> (Confirmation)	
		0.1	99	2				
		0.01	105	3	103	3		328→141 <i>m/z</i> (Confirmation)
		0.1	102	2				
Prothioconazole-6-hydroxy-Desthio*	Pollen	0.01	108	7	102	11	328→70 <i>m/z</i> (Quantification)	
		0.1	96	13				
	Nectar	0.01	96	1	96	1		328→141 <i>m/z</i> (Confirmation)
		0.1	97	1				
	Honey	0.01	101	2	101	2	328→141 <i>m/z</i> (Confirmation)	
		0.1	100	3				
		0.01	98	2	99	2		328→141 <i>m/z</i> (Confirmation)
		0.1	99	1				
1,2,4-Triazole	Pollen	0.01	89	14	94	11	70→43 <i>m/z</i> (Quantification) Hypercarb column	
		0.1	100	2				
	Nectar	0.01	98	11	100	7		70→43 <i>m/z</i> (Confirmation) Biphenyl column
		0.1	102	2				
	Honey	0.01	92	9	94	6	70→43 <i>m/z</i> (Confirmation) Biphenyl column	
		0.1	95	3				
		0.01	99	11	96	9		70→43 <i>m/z</i> (Confirmation) Biphenyl column
		0.1	94	8				
Triazole Alanine	Pollen	0.01	98	10	102	9	157→70 <i>m/z</i> (Quantification) Hypercarb column	
		0.1	106	6				
	Nectar	0.01	111	2	111	2		157→88 <i>m/z</i> (Confirmation) Hypercarb column
		0.1	111	2				
	Honey	0.01	111	4	106	6	157→88 <i>m/z</i> (Confirmation) Hypercarb column	
		0.1	102	3				
		0.01	113	2	109	5		157→88 <i>m/z</i> (Confirmation) Hypercarb column
		0.1	104	5				
Triazole Acetic Acid	Pollen	0.01	95	3	96	6	128→70 <i>m/z</i> (Quantification) Biphenyl column	
		0.1	98	7				
	Nectar	0.01	100	2	99	2		128→70 <i>m/z</i> (Quantification) Hypercarb column
		0.1	99	1				
	Honey	0.01	90	11	91	7	128→70 <i>m/z</i> (Confirmation) Hypercarb column	
		0.1	93	2				
		0.01	99	5	94	7		128→70 <i>m/z</i> (Confirmation) Hypercarb column
		0.1	89	7				
Analyte	Matrix	Fortification Level (mg/kg) (n = 5)	Mean Recovery (%)	Rel. Std. Dev. (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments	
Triazole Lactic	Pollen	0.01	110	4	108	4	158→70 <i>m/z</i>	

Acid	Nectar	0.1	105	4			(Quantification) Biphenyl column
		0.01	103	3	104	2	158→70 <i>m/z</i>
		0.1	104	1			(Quantification) Hypercarb column
	Honey	0.01	96	9	97	6	
		0.1	97	1			
		0.01	105	8	102	7	158→43 <i>m/z</i>
		0.1	98	2			(Confirmation) Hypercarb column

\* For group 2 analytes, fortification level was equivalent to prothioconazole-desthio as parent.

**Table A 25: Characteristics for the analytical method used for validation of prothioconazole residues in pollinator matrices**

	Group 1	Group 2	Group 3
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions. For both mass transitions of honey and for all mass transitions used for quantification of all other matrices the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes, therefore showing that the method is highly specific. Representative chromatograms, mass spectra and product ion spectra are provided.	LC-MS/MS determination was conducted by monitoring two mass transitions. For both mass transitions of honey and for all mass transitions used for quantification of all other matrices the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes, therefore showing that the method is highly specific. Representative chromatograms, mass spectra and product ion spectra are provided.	Honey: LC-DMS/MS/MS determination was conducted by monitoring one mass transition on two different columns. Additional monitoring of mass transition <i>m/z</i> 70 → 70 was also used for monitoring peak identity of the 1,2,4-triazole on both columns. Due to the specific selectivity with the DMS-coupled MS/MS, <i>m/z</i> 70 → 70 may be used for peak confirmation since the separation of the analyte is enhanced due to this detection technique. Pollen, nectar: LC-DMS/MS/MS determination was conducted by monitoring two mass transitions. For both mass transitions of honey and for all mass transitions used for quantification of all other matrices the samples showed no significant interference above 30 % of LOQ at the retention time of the analytes, therefore showing that the method is highly specific. Representative chromatograms, mass spectra and product ion spectra are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration. calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients R obtained were > 0.99. Please refer to table A24 below.		
Calibration range	Please refer to table A24 below.		
Assessment of matrix effects is presented	Yes (significant in pollen)	Yes (significant in pollen and honey)	Yes (significant in pollen and honey)
Limit of determination/ quantification	The LOQ is the lowest validated fortification level for each analyte (group 1 and 3) or parent equivalent (group 2) and was thus successfully established at 0.01 mg/kg in honey for the two mass transitions and one mass transition for all other matrices (pollen, nectar). The LOD was set at 0.003 mg/kg for pollen, nectar and honey, which is 30 % of the LOQ, considering parent equivalent for group 2 analytes. Lowest validated fortification level for sugar solution was 6.65 mg/kg and evaluated for one mass transition.		
Stability of standards and extracts	Stock solutions: Prothioconazole in acetonitrile stable for at least 41 days at 1 °C to 10 °C. Prothioconazole-desthio stable in acetonitrile for at least 41 days when at 1 °C to 10 °C. PTZ- $\alpha$ -hydroxy-desthio stable in acetonitrile for at least 120 at 1 °C to 10 °C. PTZ-3-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C. PTZ-4-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C.		

	Group 1	Group 2	Group 3
	<p>PTZ-5-hydroxy-desthio stable in acetonitrile for at least 120 days at 1 °C to 10 °C. PTZ-6-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C. All analytes of group 3 stable in HPLC water for at least 76 at 1 °C to 10 °C.</p> <p>Extracts: Almost all analytes were found to be stable in final extracts of all matrices for at least 7 days when stored at typically 1 °C to 10 °C in the dark. However, samples of pollen containing prothioconazole should be analysed as quickly as possible after extraction, since first determination of extract stability showed low recovery after 6 days. A second analysis in study S20-09716 showed good recoveries and a stability over 15 days. In case a degradation occurs it would be safe to analyse the samples latest in the first few days after extraction.</p>		

**Table A 26: Linearity of detector response**

Analyte	Matrix/Transition	Calibration range	Equation	r
Prothioconazole	Pollen 344→154 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.22e+005 x + 1.02e+004	0.9999
	Nectar 344→154 m/z (Quantification)	0.03 – 3 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 3.23e+005 x + 264	0.9999
	Sugar solution 344→189 m/z (Quantification)	1 – 60 ng/mL (1.68 – 101 mg/kg)* n = 6	y = 5.96e+004 x + 2.14e+003	0.9998
Prothioconazole-desthio	Honey 312→125 m/z (Quantification)	0.06 – 5 ng/mL (0.003 – 0.25 mg/kg) n = 6	y = 1.33e+006 x + 1.33e+003	1.0000
	Honey 312→70 m/z (Confirmation)	0.06 – 5 ng/mL (0.003 – 0.25 mg/kg) n = 6	y = 1.9e+006 x -3.8e+003	1.0000
	Pollen 312→125 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 9.27e+005 x + 2.5e+004	0.9998
	Nectar 312→125 m/z (Quantification)	0.03 – 3 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.37e+006 x + 1.45e+003	0.9996
Prothioconazole-alpha-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 3.2e+005 x -451	1.0000
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.04e+005 x -336	1.0000
	Nectar 328→70 m/z (Quantification)	0.075 – 7 ng/mL (0.003 – 0.28 mg/kg) n = 6	y = 3.21e+005 x + 2.2e+003	0.9999
	Pollen 328→70 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 7.43e+004 x -998	0.9999
Prothioconazole-3-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 2.31e+005 x -24.7	0.9999
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.23e+005 x + 659	0.9999
	Nectar 328→70 m/z (Quantification)	0.075 – 7 ng/mL (0.003 – 0.28 mg/kg) n = 6	y = 2.22e+005 x + 1.47e+003	0.9999
	Pollen 328→70 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 5.5e+004 x -97.2	0.9996
Prothioconazole-4-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 2.03e+005 x -51.6	1.0000
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.94e+005 x + -552	0.9999
	Nectar 328→70 m/z (Quantification)	0.075 – 7 ng/mL (0.003 – 0.28 mg/kg) n = 6	y = 1.97e+005 x + 859	0.9999
	Pollen 328→70 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 4.97e+004 x -28.9	1.0000
Prothioconazole-5-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 2.11e+005 x -1.86e+003	1.0000
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 1.1e+005 x + 3.95	1.0000
	Nectar 328→70 m/z (Quantification)	0.075 – 7 ng/mL (0.003 – 0.28 mg/kg) n = 6	y = 2.05e+005 x + 286	0.9998
	Pollen 328→70 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 6.82e+004 x + 1.91e+003	0.9991
Prothioconazole-6-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	y = 3.31e+005 x -147	0.9999

Analyte	Matrix/Transition	Calibration range	Equation	r
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	$y = 2.37e+005 x + 821$	0.9998
	Nectar 328→70 m/z (Quantification)	0.075 – 7 ng/mL (0.003 – 0.28 mg/kg) n = 6	$y = 3.3e+005 x + 1.93e+003$	0.9999
	Pollen 328→70 m/z (Quantification)	0.1– 10 ng/mL (0.003 – 0.3 mg/kg) n = 6	$y = 7.6e+004 x -1.71e+003$	0.9984
1,2,4-Triazole	Honey 70→43 m/z (Quantification) Hypercarb column	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 9.82e+003 x + 165$	0.9998
	Honey 70→43 m/z (Confirmation) Biphenyl column	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 805 x + 92$	0.9981
	Pollen 70→43 m/z (Quantification)	0.09 – 7.5 ng/mL (0.003 – 0.25 mg/kg) n = 7	$y = 5.85e+003 x + 341$	0.9987
	Nectar 70→43 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 7	$y = 2.17e+004 x + 290$	0.9997
Triazole Alanine	Honey 157→70 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 9.89e+003 x + 812$	0.9994
	Honey 157→88 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 3.99e+003 x + 648$	0.9980
	Pollen 157→70 m/z (Quantification)	0.09 – 7.5 ng/mL (0.0003 – 0.25 mg/kg) n = 7	$y = 1.55e+004 x + 1.89e+003$	0.9996
	Nectar 157→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 7	$y = 8.96e+004 x -279$	0.9999
Triazole Acetic Acid	Honey 128→70 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 2.57e+004 x + 5.22e+003$	0.9995
	Honey 128→43 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 1.19e+003 x + 268$	0.9976
	Pollen 128→70 m/z (Quantification)	0.09 – 7.5 ng/mL (0.0003 – 0.25 mg/kg) n = 7	$y = 2.69e+004 x + 2.53e+003$	0.9999
	Nectar 128→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 7	$y = 1.88e+005 x -365$	0.9999
Triazole Lactic Acid	Honey 158→70 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 2.63e+004 x + 1.79e+003$	0.9998
	Honey 158→43 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 7	$y = 3.94e+003 x + 78.9$	0.9997
	Pollen 158→70 m/z (Quantification)	0.09 – 7.5 ng/mL (0.0003 – 0.25 mg/kg) n = 7	$y = 2.86e+004 x + 7.06e+003$	0.9996
	Nectar 158→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 7	$y = 1.47e+005 x -44.7$	1.0000

\*density (sugar solution) = 1.19 g/mL

## Conclusion

The method validation is considered valid and acceptable according SANTE/2020/12830, Rev.1 for the determination of prothioconazole, prothioconazole-desthio (Group1), prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio (Group 2), as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in pollinator matrices.

### A 2.1.2.2.1.2 Independent laboratory validation

Comments of zRMS:	The analytical method has been independently validated according to the guidance document SANTE/2020/12830, rev. 1 for the determination of prothioconazole-desthio (Group1), prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio (Group 2) and 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid (Group 3) in honey. An LOQ of 0.01 mg/kg was confirmed for all analytes in honey. The study is acceptable.
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Reference: KCP 5.2/02

Report Independent Laboratory Validation of Analytical Methods for the Determination of

Prothioconazole Metabolites in Honey, Greiner M., 2021, S21-02654

Guideline(s): SANTE/2020/12830, rev.1

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

Methods were applied as in KCP 5.2/01 Kalathoor R., 2021, Report No. S20-0974. No addition or modification to the original method other than optimization of instrumental parameters was done for analytes of group 1 and 2.

For the determination of 1,2,4-Triazole (T) and Triazole Acetic Acid (TAA) (group 3) in honey, the following minor modifications to the original method were made:

- Confirmation of 1,2,4-Triazole (T) values was performed using the “pseudo multi reaction monitoring (MRM) transition”  $m/z$  70→70 in combination with the hypercarb column. Chromatograms of 1,2,4-Triazole (T) samples using the biphenyl column for confirmation could not be evaluated due to strong matrix interferences.
- Due to insufficient sensitivity of the confirmation mass transition  $m/z$  128→43 of Triazole Acetic Acid (TAA), confirmation was performed using a second chromatographic method. The mass transition  $m/z$  128→70 was also used for confirmation in combination with a biphenyl column instead of the mass transition  $m/z$  128→43 in combination with a hypercarb column.

These changes had no impact on the study and are fully compliant with SANTE/2020/12830, rev.1.

## Results and discussions

**Table A 27: Recovery results from independent laboratory validation of prothioconazole and its metabolites using the analytical method**

Analyte	Matrix	Fortification Level (mg/kg) (n = 5)	Mean Recovery (%)	Rel. Std. Dev. (%)	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Comments
Prothioconazole-desthio	Honey	0.01	102	2.0	102	1.7	312→125 $m/z$ (Quantification)
		0.1	102	1.7			
		0.01	104	2.0	103	2.1	312→70 $m/z$ (Confirmation)
		0.1	102	1.8			
Prothioconazole-alpha-hydroxy-Desthio*	Honey	0.01	107	5.6	105	4.7	328→70 $m/z$ (Quantification)
		0.1	103	2.4			
		0.01	106	6.4	104	4.9	328→141 $m/z$ (Confirmation)
		0.1	103	2.9			
Prothioconazole-3-hydroxy-Desthio*	Honey	0.01	105	6.4	103	4.7	328→70 $m/z$ (Quantification)
		0.1	102	2.1			
		0.01	109	6.0	105	6.2	328→141 $m/z$ (Confirmation)
		0.1	102	4.1			
Prothioconazole-4-hydroxy-Desthio*	Honey	0.01	110	6.2	107	5.7	328→70 $m/z$ (Quantification)
		0.1	103	2.2			
		0.01	108	4.4	105	4.3	328→141 $m/z$ (Confirmation)
		0.1	103	2.3			
Prothioconazole-5-hydroxy-Desthio*	Honey	0.01	110	5.5	106	5.2	328→70 $m/z$ (Quantification)
		0.1	103	1.8			
		0.01	104	4.4	104	3.7	328→141 $m/z$ (Confirmation)
		0.1	103	3.2			
Prothioconazole-6-hydroxy-Desthio*	Honey	0.01	107	5.4	105	4.5	328→70 $m/z$ (Quantification)
		0.1	103	2.5			
		0.01	107	5.1	106	3.9	328→141 $m/z$ (Confirmation)
		0.1	104	1.8			
1,2,4-Triazole	Honey	0.01	100	6.5	100	5.0	70→43 $m/z$ (Quantification) Hypercarb column
		0.1	100	3.6			
		0.01	102	4.4	101	4.5	70→70 $m/z$ (Confirmation)
		0.1	101	5.0			

							Hypercarb column
<b>Triazole Alanine</b>	Honey	0.01	100	12	100	8.6	157→70 m/z (Quantification) Hypercarb column
		0.1	100	4.9			
		0.01	98.0	12	96.6	9.2	157→88 m/z (Confirmation) Hypercarb column
		0.1	95.2	5.2			
<b>Triazole Acetic Acid</b>	Honey	0.01	93.3	2.3	94.8	2.9	128→70 m/z (Quantification) Hypercarb column
		0.1	96.4	2.7			
		0.01	95.3	3.9	94.3	9.1	128→70 m/z (Confirmation) Biphenyl column
		0.1	93.2	13			
<b>Triazole Lactic Acid</b>	Honey	0.01	98.4	8.5	94.8	7.9	158→70 m/z (Quantification) Hypercarb column
		0.1	91.2	5.6			
		0.01	95.4	13	95.0	9.8	158→43 m/z (Confirmation) Hypercarb column
		0.1	94.5	6.0			

\*Expressed as PTZ-desthio

**Table A 28: Characteristics for the analytical method used for validation of prothioconazole residues in pollinator matrices**

	Group 1	Group 2	Group 3
Specificity	Quantification was performed by use of LC-MS/MS detection for analytes of group 1 and 2 and LC-DMS-MS/MS detection for analytes of group 3. Two mass transitions were evaluated in order to demonstrate that the methods achieve a high level of selectivity, except for triazole alanine (TA). For triazole alanine (TA) a second chromatographic technique was used for confirmation of peak identity. No significant interference above 30 % of LOQ was detected in any of the control sample extracts of honey, so that a high level of selectivity was demonstrated. Representative chromatograms, mass spectra and product ion spectra are provided.		
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients R obtained were > 0.99. Please refer to table A27 below.		
Calibration range	Please refer to table A27 below.		
Assessment of matrix effects is presented	Yes (insignificant in honey)	Yes (insignificant in honey)	Yes (significant in honey)
Limit of determination/quantification	The LOQ is the lowest validated fortification level for each analyte (group 1 and 3) or parent equivalent (group 2) and was thus successfully established at 0.01 mg/kg in honey for two mass transitions each analyte. The LOD was set at 0.003 mg/kg, which is 30 % of the LOQ, considering parent equivalent for group 2 analytes.		
Stability of standards and extracts	<p>Standard solutions:</p> <p>PTZ-desthio was found to be stable for at least 34 days when prepared in acetonitrile or methanol/water (40/60, v/v) containing 50 g/L cysteine hydrochloride and stored at typically 1 °C to 10 °C in the dark.</p> <p>PTZ-alpha-hydroxy-desthio, PTZ-3-hydroxy-desthio, PTZ-4-hydroxy-desthio, PTZ-5-hydroxy-desthio and PTZ-6-hydroxy-desthio were found to be stable for at least 41 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark</p> <p>PTZ-alpha-hydroxy-desthio, PTZ-3-hydroxy-desthio, PTZ-4-hydroxy-desthio, PTZ-5-hydroxy-desthio and PTZ-6-hydroxy-desthio were found to be stable for at least 37 days when prepared in acetonitrile/water/10mM ammonium formate (1/1/2, v/v/v) solution and stored at typically 1 °C to 10 °C in the dark.</p> <p>1,2,4-Triazole (T), Triazole Alanine (TA), Triazole Acetic Acid (TAA) and Triazole Lactic Acid (TLA) were found to be stable for at least 9 days when prepared in water and stored at typically 1 °C to 10 °C in the dark.</p> <p>Extracts:</p> <p>PTZ-desthio was found to be stable in final extracts of honey for at least 19 days when stored at typically 1 °C to 10 °C in the dark.</p> <p>PTZ-alpha-hydroxy-desthio, PTZ-3-hydroxy-desthio, PTZ-4-hydroxy-desthio, PTZ-5-hydroxy-desthio</p>		

	Group 1	Group 2	Group 3
	and PTZ-6-hydroxy-desthio were found to be stable in final extracts of honey for at least 36 days when stored at typically 1 °C to 10 °C in the dark.		
	1,2,4-Triazole (T), Triazole Alanine (TA), Triazole Acetic Acid (TAA) and Triazole Lactic Acid (TLA) were found to be stable in final extracts of honey for at least 13 days when stored at typically 1 °C to 10 °C in the dark.		

**Table A 29: Linearity of detector response**

Analyte	Matrix/Transition	Calibration range	Equation	r
Prothioconazole-desthio	Honey 312→125 m/z (Quantification)	0.06 – 5 ng/mL (0.003 – 0.25 mg/kg) n = 6	y = 3.14e+006 x + 2.72e+004	0.9999
	Honey 312→70 m/z (Confirmation)	0.06 – 5 ng/mL (0.003 – 0.25 mg/kg) n = 6	y = 3.22e+006 x + 1.69e+004	0.9999
Prothioconazole-alpha-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 1.69e+005 x – 3.69e+003	0.9998
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 1.29e+005 x – 2.17e+003	0.9998
Prothioconazole-3-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 9.51e+004 x – 2.14e+003	0.9998
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 4.28e+004 x – 954	0.9996
Prothioconazole-4-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 1.81e+005 x – 4.73e+003	0.9999
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 2.58e+005 x – 6.54e+003	0.9999
Prothioconazole-5-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 1.64e+005 x – 4.53e+003	0.9999
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 1.38e+005 x – 2.97e+003	0.9998
Prothioconazole-6-hydroxy-Desthio	Honey 328→70 m/z (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 2.53e+005 x – 7.02e+003	0.9999
	Honey 328→141 m/z (Confirmation)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 8	y = 2.84e+005 x – 6.47e+003	0.9998
1,2,4-Triazole	Honey 70→43 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 1.95e+004 x + 1.98e+003	0.9983
	Honey 70→70 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 1.59e+005 x + 2.25e+004	0.9992
Triazole Alanine	Honey 157→70 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 5.72e+003 x + 296	0.9993
	Honey 157→88 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 2.64e+003 x + 31.8	0.9954
Triazole Acetic Acid	Honey 128→70 m/z (Hypercarb column) (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 4.7e+004 x + 3.64e+003	0.9999
	Honey 128→70 m/z (Biphenyl column) (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 1.66e+005 x – 5.09e+003	0.9989
Triazole Lactic Acid	Honey 158→70 m/z (Quantification)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 4.75e+004 x – 3.5e+003	0.9994
	Honey 158→43 m/z (Confirmation)	0.15 – 7.5 ng/mL (0.003 – 0.15 mg/kg) n = 6	y = 7.27e+003 x – 416	0.9982

## Conclusion

The primary analytical method is considered independently validated and acceptable according SANTE/2020/12830, Rev.1 for the determination of prothioconazole-desthio (Group1), prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio (Group 2), as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in honey.

No addition or modification to the original method (KCP 5.2/01 Kalathoor R., 2021, Report No.S20-09747) other than optimization of instrumental parameters was done for analytes of group 1 and 2.

For the determination of 1,2,4-Triazole (T) and Triazole Acetic Acid (TAA) (group 3) in honey, the following minor modifications to the original method were made:

- Confirmation of 1,2,4-Triazole (T) values was performed using the “pseudo multi reaction monitoring (MRM) transition”  $m/z$  70→70 in combination with the hypercarb column. Chromatograms of 1,2,4-Triazole (T) samples using the biphenyl column for confirmation could not be evaluated due to strong matrix interferences.
- Due to insufficient sensitivity of the confirmation mass transition  $m/z$  128→43 of Triazole Acetic Acid (TAA), confirmation was performed using a second chromatographic method. The mass transition  $m/z$  128→70 was also used for confirmation in combination with a biphenyl column instead of the mass transition  $m/z$  128→43 in combination with a hypercarb column.

These changes had no impact on the study and are fully compliant with SANTE/2020/12830, rev.1.

No communication with the method developers or others familiar with the method was necessary to carry out the analysis.

#### **A 2.1.2.2.1.3 Confirmatory method (if required)**

Confirmatory data presented in initial method validation.

#### **A 2.1.2.2.1.4 Extraction efficiency**

According to SANTE 2017/10632 Rev. 3 22 November 2017, Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods, for new product authorisations for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. In case this document did not yet apply, when the data for the latest renewal or approval were submitted, at this stage no new studies or data related to extraction efficiency are required. This means in practice that for new product authorisations for which no new MRL is required, no additional proof of extraction efficiency will be required.

Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

#### **A 2.1.2.7 A.2.A.9 Other Studies/ Information**

No new or additional studies have been submitted.