

REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: CA3642

Product name(s): JOUST PRO

Chemical active substance:

Prothioconazole, 150 g/L

Azoxystrobin, 150 g/L

Central zone

Zonal Rapporteur Member State: PL

CORE ASSESSMENT

New Authorisation (Art.33)

Sponsor: Nufarm Crop Products UK Limited

Applicant: Nufarm Polska Sp. z o. o.

Submission date: 01/02/2023; updated July 2023, November 2023, September 2024

MS Finalisation date: December 2023, update October 2024
(initial Core Assessment)

December 2024 (final Core Assessment)

Version history

When	What
February 2023	First submission
July 2023	Update following zRMS PL request on June 22 nd , 2023
November 2023	Additional update following zRMS PL request on June 22 nd , 2023
December 2023	<p>Initial zRMS assessment</p> <p>The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are struck through and shaded for transparency.</p>
October 2024	<p>Core Assessment updated following the commenting period and adding an assessment of new studies</p> <p>Additional information/assessments included by the zRMS in the report in response to comments received from the cMS and the Applicant are highlighted in yellow. Not agreed or not relevant information are struck through and shaded for transparency.</p>
December 2024	<p>Final report (Core Assessment updated following the third commenting period)</p> <p>Additional information/assessments included by the zRMS in the report in response to comments received from the cMS and the Applicant are highlighted in yellow. Not agreed or not relevant information are struck through and shaded for transparency.</p>

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5 Analytical methods

5.1 Conclusion and summary of assessment

According to SANTE/11509/2013– rev. 5.2 and since the active substance azoxystrobin is not yet renewed (AIR4), the “old data requirements” (Reg. (EU) No 544/2011) and the endpoints from the previous monograph of azoxystrobin (DAR, 2009) apply to the current assessment. Studies from the DAR are not protected anymore.

The applicant Nufarm has a letter of co-ownership by the Azoxystrobin Task Force which authorizes Nufarm to access to the studies submitted during the AIR4 renewal of azoxystrobin (process currently ongoing).

According to SANTE/11509/2013– rev. 5.2 and since the active substance prothioconazole is not yet renewed (AIR3), the “old data requirements” (Reg. (EU) No 544/2011) and the endpoints from the previous monograph of prothioconazole (DAR, 2005) apply to the current assessment. Studies from the DAR are not protected anymore.

zRMS conclusions:

Prothioconazole

The endpoints reported in EFSA Scientific Report (2007) 106 are still valid for the ongoing evaluations.

However, taking into account conclusions EFSA regarding residue definitions presented in EFSA Journal 2020;18(2):5999, EFSA Journal 2014;12(5):3689 and EFSA Journal 2018;16(7):5376, based on the metabolic pattern identified in metabolism studies, hydrolysis studies, the toxicological significance of metabolites and degradation products, the residue definitions for plant products were proposed as ‘prothioconazole-desthio (sum of isomers)’ for enforcement and, as follows, for the risk assessment:

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

Since all compounds included in the residue definitions are a mixture of enantiomers and since there are no enantiospecific analytical methods, the residue definitions are expressed as “sum of isomers”.

Although the residue definition for risk assessment includes consideration of all metabolites containing a common moiety, it is not possible to develop a common moiety method to meet the residue definition for risk assessment. For this reason, all the analytes have to be determined separately. 6 analytes, representing the major portion of the TRR (Total Radioactive Residue) for prothioconazole in the plant metabolism studies, should be determined in residue trials. These are: prothioconazole-desthio, 3-hydroxy-prothioconazole-desthio, 4-hydroxy-prothioconazole-desthio, 5-hydroxy-prothioconazole-desthio, 6-hydroxy-prothioconazoledesthio and alpha-hydroxy-prothioconazole-desthio (including all their acid-hydrolysable conjugates).

The residue definition for enforcement in animal products was set as prothioconazole-desthio (sum of isomers) for all the livestock matrices (EFSA Journal 2014;12(5):3689).

For risk assessment, the residue was defined in all commodities of animal origin as the sum of prothioconazole-desthio and all metabolites containing the 2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl-2H-1,2,4-triazole moiety, expressed as prothioconazole-desthio (sum of isomers).

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

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

EFSA Scientific Report (2007):

Analytical methods for residues (Annex IIA, point 4.2)

Food/feed of plant origin (principle of method and LOQ for methods for monitoring purposes)	Weeren, Pelz 2000 (GC-MS, JAU6476-desthio) LOQ Wheat, Barley (Forage, Straw): 0.05 mg/kg LOQ Wheat, Barley (Grain), Canola (Seed), Tomato, Orange (Fruit): 0.02 mg/kg
Food/feed of animal origin (principle of method and LOQ for methods for monitoring purposes)	Heinemann 2001b (HPLC-MS/MS, JAU6476-desthio, JAU6476-3 hydroxy-desthio, JAU6476-4-hydroxy-desthio) LOQ Milk: 0.004 mg/kg LOQ Meat, Liver, Kidney, Fat: 0.01 mg/kg Open: there is no method available for the glucuronide conjugate
Soil (principle of method and LOQ)	Schramel 2000 (HPLC-MS/MS, JAU6476, JAU6476-desthio, JAU6476-S-methyl*) * for monitoring not needed LOQ Soil: 0.006 mg/kg Add'l method: Steinhauer 2001 (GC-MS, JAU6476-desthio) LOQ Soil: 0.01 mg/kg
Water (principle of method and LOQ)	Sommer 2001b (HPLC-MS/MS, JAU6476, JAU6476-desthio) LOQ Surface and Drinking water: 0.1 µg/L for JAU6476 and 0.05 µg/L for JAU6476-desthio
Air (principle of method and LOQ)	Maasfeld 2002a (HPLC-MS/MS, JAU6476) LOQ Air: 0.015 mg/m ³ Additional method: Maasfeld 2002b (HPLC-MS/MS, JAU6476-desthio) LOQ Air: 0.0006 mg/m ³
Body fluids and tissues (principle of method and LOQ)	Open, data will be required if ECB classify the active as toxic

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

Methods for enforcement of residues in food of plant origin

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

The multi-residue QuEChERS method in combination with HPLC-MS/MS, as described by CEN (2008), is also available to analyse the prothioconazole-desthio in plant commodities. Nevertheless, the validation data reported are too limited to conclude on the validity of this analytical method (EURL, 2013).

Hence it is concluded that prothioconazole-desthio can be enforced in food of plant origin with an LOQ of 0.02 mg/kg in high oil content and dry commodities and an LOQ of 0.05 mg/kg in high water content commodities and in straw taking into account the highest LOQ of both methods.

Methods for enforcement of residues in food of animal origin

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

The available analytical method is not enantioselective, hence the sum of isomers will be analyzed.

The Applicant submitted a number of methods for analysis of residues of prothioconazole for the generation of pre-authorization data and methods for post-authorization control and monitoring purposes.

The details of the evaluation of new and additional studies are referred in Appendix 2.

Since many MRLs have been lowered to 0.01 mg/kg, the validated LOQ of the EU agreed methods by Weeren and Pelz (2000) and Class (2001) are not sufficient to monitor these lowered MRLs for food of plant origin. To cover the current residue definition and MRL limits, the Applicant 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.

The 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:

zRMS-PL shares the submitted explanation of the methods. 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 Pro (cereals and oilseeds).

Additionally, 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 (data gap).

According to information from the Applicant, the earliest date for obtaining/submitting a description of the method is the beginning of 2024.

The applicant provided the ILV of S16-04434 (Heinz N., 2024, S23-106298) for the determination of relevant residues of prothioconazole and prothioconazole-desthio in/on matrices of plant origin (high water content, high acid, high oil content and high protein/high starch content) by HPLC-MS/MS with LOQ of 0.01 mg/kg. The ILV is acceptable. The analytical method S16-04434 was successfully independently validated. The details of the evaluation of new study is referred in Appendix 2.

Note:

1. 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.

A body fluids method for prothioconazole-desthio was submitted by Bayer and was evaluated within the framework of the active substance renewal. The limit of quantification was established at 0.05 mg/L, expressed as prothioconazole-desthio, but according to the SANTE/2020/12830, Rev.2, 14. February 2023, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues.

In zRM-PL opinion, it is necessary to supply the method for determining the residues of prothioconazole in body fluids with lower LOQ=0.01 mg/L at the renewal of the active substance and/or re-evaluation of plant production product (data gap).

2. According to the conclusions presented in EFSA Journal 2014;12(5):3689, a fully validated analytical method for the determination of prothioconazole-desthio in eggs is required.

Applicant submitted the analytical method 01009 for the determination of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxydesthio, and JAU 6476-4,5-dihydroxy-desthio in/on matrices of animal origin: milk, muscle, kidney, liver, fat and egg with LOQ 0.01 mg/kg. The BCS Analytical Method No. 010091 has been independently validated.

No additional data are required.

3. 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.2 14. February 2023, the ILV for drinking water should be submitted (data gap).

Applicant submitted the HPLC-MS/MS analytical method (Krebber, R.; Sandau, C., 2015, Report No. M-526061-01-1) with its ILV (Thies, S., 2015, Report No. M-536990-01-1) for the determination of prothioconazole and prothioconazole-desthio in surface water with LOQ of 0.05 µg/L (prothioconazole and prothioconazole-desthio). The method is also applicable for drinking water.

In our opinion, 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 should be provided as a post-registration requirement.

The applicant provided the ILV (Heinz N., 2024, S23-106298) of the method S16-04434 for the determination of relevant residues of prothioconazole and prothioconazole-desthio in/on matrices of plant origin by HPLC-MS/MS with LOQ of 0.01 mg/kg. The ILV is acceptable.

It is necessary to supply the above-mentioned method for determining the residues of prothioconazole in body fluids and tissues at the renewal of the active substance and/or re-evaluation of plant production product.

Azoxystrobin

The methods available for azoxystrobin in plant and animal matrices were sums in EFSA document “Review of the existing MRLs for azoxystrobin” (EFSA Journal 2013;11(12):3497):

“1. Methods for enforcement of residues in food of plant origin

During the renewal peer review under Directive 91/414/EC, the multi-residue method DFG S 19 using HPLC-MS/MS and its ILV were evaluated and validated in plant matrices for the determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in dry (cereals grain), acidic (orange), high water content (lettuce) and high oil content (oilseed rape) commodities (United Kingdom, 2009a, 2009b; FAO, 2008).

Furthermore, an analytical method using HPLC-MS/MS and its ILV were evaluated and adequately validated in plant matrices for the determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in dry (wheat, barley grain), acidic (grape, mandarin, orange), high water content (tomato, lettuce, cabbage, carrot, kale, potato) and high oil content (avocado, sunflower seed, oilseed rape,) commodities, and in hops (United Kingdom, 2009b; FAO, 2008). The multi-residue QuEChERS methods in combination with HPLC-MS/MS and GC/MS, as described by CEN (2008), are also available to analyse parent azoxystrobin but validation data were not evaluated in detail because a validated analytical method is reported above.

Hence, it is concluded that parent azoxystrobin can be enforced in food of plant origin with an LOQ of 0.01 mg/kg in dry, acidic, high water content and high oil content commodities, and in hops. As the active substance does not contain a chiral center, the analytical method is considered as specific to the active substance.

2. Methods for enforcement of residues in food of animal origin

During the peer review under Directive 91/414/EEC, an analytical method using GC-NPD and its ILV were evaluated and validated for determination of parent azoxystrobin with an LOQ of 0.001 mg/kg in milk and 0.01 mg/kg in eggs, liver, fat, muscle. Nevertheless, no confirmatory method was available (United Kingdom, 2009a; FAO, 2008).

Furthermore, an analytical method using HPLC-MS/MS and its ILV were evaluated in the JMPR report and validated in food of animal origin for determination of parent azoxystrobin with an LOQ of 0.01 mg/kg in muscle, fat, milk, kidney, liver and eggs (FAO, 2008).

Hence, it is concluded, that parent azoxystrobin can be enforced in food of animal origin with an LOQ of at least 0.01 mg/kg in muscle, fat, milk, kidney, liver and eggs.”

Therefore, no further consideration of monitoring methods for plant and animal matrices is necessary.

In “Peer Review of the pesticide risk assessment of the active substance azoxystrobin” (EFSA Journal 2010; 8(4):1542) it is stated that “Monitoring of residues of azoxystrobin in groundwater, drinking water and surface water can be done by GC-MSD. Pending on the data gap identified in section 4, the residue definition for water might change and therefore further methods could be required in the future. Adequate methods are available for the determination of residues of azoxystrobin in soil and air.”

The Applicant submitted a number of methods for analysis of residues of azoxystrobin 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.

No additional data for azoxystrobin is required to support this application.

Sufficiently sensitive and selective analytical methods are available for the active substances prothioconazole and azoxystrobin, and relevant impurities (prothioconazole-desthio, Z-azoxystrobin and toluene) in the plant protection product.

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

Noticed data gaps are:

~~—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, Rev.2, 14. February 2023 and should be provided as a post registration requirement;~~
- an analytical method for the determination of prothioconazole in body fluids with lower LOQ=0.01 mg/L is required according to SANTE/2020/12830, Rev.2, 14. February 2023 and should be provided at the renewal of the active substance and/or re-evaluation of plant production product.

Commodity/crop	Supported/ Not supported
Dry commodity/Wheat, Triticale, Rye, Oat, Barley, Flax	Supported
High Oil Commodity/Oilseed Rape, Sunflower, Linseeds, Poppy seeds, Mustard, Gold of pleasure	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 substances 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 and azoxystrobin in plant protection product is provided as follows:

Comments of zRMS:	The proposed analytical method was successfully validated for the determination of active substances in plant protection product according to the requirements laid down by SANCO3030/99 rev.5.
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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 150 g/L + Azoxystrobin 150 g/L SC, CA3642, Wang, Q., 2022, Report No. ABC-2021-018
Guideline(s):	EU SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples of CA3642 (50 mg) are transferred in a 50mL volumetric flask, dispersed with 1 mL of water and made up to the volume with Acetonitrile. The samples are analysed for prothioconazole and azoxystrobin content by HPLC-UV, using an Eclipse Plus C18 column (100 mm x 4.6 mm, 3.5 µm). Calibration standards are prepared independently by dissolving amounts of the prothioconazole reference item and azoxystrobin reference item into a 50mL volumetric flask. 1 mL of water was added into the volumetric flask and the volume was made up to 50mL with acetonitrile. Each standard solution were injected twice in HPLC-UV system.

Analytical conditions

System: Agilent 1200 HPLC system with DAD
Column: Eclipse Plus C18 column (100 mm x 4.6 mm, 3.5 µm)
Mobile phase A: water (0.1% H₃PO₄)
Mobile phase B: Acetonitrile
Isocratic: A/B 45/55 v/v at 0.5 mL/min
Column temperature: 30°C
Injection volume: 3 µL (needle wash mode)
Detection: UV at 210 nm
Run time: 12.0 min
Retention time:
 Azoxystrobin: 7.5 min
 Prothioconazole: 10.7 min

Quantitation of Azoxystrobin (or Prothioconazole) was performed by the following equation:

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

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

Where:

A = the average peak area ratio of Active Ingredient in Test Item solution

A' = the average peak area ratio of Active Ingredient in Azoxystrobin (or Prothioconazole) standard solution;

W = the mass of Test Item;

W' = the mass of Azoxystrobin (or Prothioconazole) standard;

P = the purity of Azoxystrobin (or Prothioconazole) standard

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance prothioconazole in plant protection product CA3642

	Prothioconazole			
Author(s), year	Wang, Q. (2022)			
Principle of method	HPLC-DAD(210nm)			
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of detector response was demonstrated injecting six concentrations (injected in duplicate) of reference standard in the approximate range of 14.90 to 399.0 mg/L of prothioconazole (equivalent to 1.49% w/w – 39.9% w/w prothioconazole in the test item). $y = 14.3965 x + 248.8122$ Correlation coefficient: R = 0.9983			
		mg pure prothioconazole in L acetonitrile	% w/w pure prothioconazole in product CA3642*	g prothioconazole in L product CA 3642
	Lower standard	14.9 mg/L	1.49% w/w	16.4 g/L
	Higher standard	399.0 mg/L	39.9% w/w	439.1 g/L
	Nominal*	135 mg/L	13.5% w/w	148.5 g/L
	± 20% of nominal conc.	108-162 mg/L	10.8-16.2%	119 – 178 g/L
	* Considering a test item solution at 1 g test item / L acetonitrile (50 mg in 50 mL) ** Considering a density of 1.1004 for product CA3642			
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability (precision) was determined from single determinations of five samples of CA3642. - Mean content: 13.83% w/w - RSD: 0.29% - RSDr: 1.80% - Hr (Horrat value): 0.16 Intermediate precision was done by another analyst on another day - Mean content: 13.84% w/w - RSD: 0.22% - RSDr: 1.80% - Hr (Horrat value): 0.12			
Accuracy n = 5 (% Recovery)	Accuracy was determined from blank sample spiked at 2 different levels (injected 5 times, n = 5) Level 1 (approx. 90% of nominal concentration) - Mean recovery = 101.34% Level 2 (approx. 110% of nominal concentration) - Mean recovery = 100.33 % The mean recoveries are within 97-103%, the accuracy is acceptable.			
Interference/ Specificity	Blank solution, 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.			
Comment	-			

Table 5.2-2: Methods suitable for the determination of active substance Azoxystrobin in plant protection product CA3642

	Azoxystrobin
Author(s), year	Wang, Q. (2022)

	Azoxystrobin			
Principle of method	HPLC-DAD(210nm)			
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of detector response was demonstrated injecting six concentrations (injected in duplicate) of reference standard in the approximate range of 15 to 400 mg/L of azoxystrobin (equivalent to 1.5% w/w – 40.0% w/w azoxystrobin in the test item).			
	y = 36.2400 x + 530.4363			
	Correlation coefficient: R = 0.9990			
		mg pure azoxystrobin in L acetonitrile	% w/w pure azoxystrobin in product CA3642*	g azoxystrobin in L product CA 3642
	Lower standard	15 mg/L	1.5% w/w	16.5 g/L
	Higher standard	400 mg/L	40.0% w/w	440.1 g/L
	Nominal*	135 mg/L	13.5% w/w	148.6 g/L
	± 20% of nominal conc.	108-162 mg/L	10.8-16.2%	119 – 178 g/L
	* Considering a test item solution at 1 g test item / L acetonitrile (50 mg in 50 mL)			
	** Considering a density of 1.1004 for product CA3642			
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability (precision) was determined from single determinations of five samples of CA3642. - Mean content: 14.06% w/w - RSD: 0.36% - RSDr: 1.80% - Hr (Horrat value): 0.20 Intermediate precision was done by another analyst on another day - Mean content: 14.07% w/w - RSD: 0.28% - RSDr: 1.80% - Hr (Horrat value): 0.16			
Accuracy n = 5 (% Recovery)	Accuracy was determined from blank sample spiked at 2 different levels (injected 5 times, n = 5) Level 1 (approx. 90% of nominal concentration) - Mean recovery = 100.84% Level 2 (approx. 110% of nominal concentration) - Mean recovery = 100.60 % The mean recoveries are within 97-103%, the accuracy is acceptable.			
Interference/ Specificity	Blank solution, 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.			
Comment	-			

Conclusion

The validation of the method for analysis of prothioconazole and azoxystrobin in CA3642 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 and azoxystrobin in CA3642.

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

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

Comments of zRMS:	The analytical method was successfully validated for the determination of the relevant impurities in the plant protection product according to the requirements laid down by SANCO3030/99 rev.5.
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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 150 g/L + Azoxystrobin 150 g/L SC, CA3642, Wang, Q., 2022, Report No. ABC-2021-018
Guideline(s):	EU SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples of CA3642 (100 mg) are transferred in 10mL volumetric flask. 0.2mL of water are added and the volume is completed to 10mL with acetonitrile. The samples are analysed for prothioconazole-desthio content by HPLC-MS, using an Eclipse Plus C8 column (100 mm x 4.6 mm, 3.5 µm). The samples are analysed for z-azoxystrobin content by HPLC-UV using an Eclipse Plus C18 (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 water and acetonitrile.

Analytical conditions

Prothioconazole-desthio (impurity 1) - HPLC/MS

System: Agilent 1200/6130 Single Quadrupole LC/MS system

Column: Eclipse Plus C8, 100 mm x 4.6 mm, 3.5 µm

Mobile phase A: water (0.05% v/v formic acid)

Mobile phase B: Acetonitrile

Gradient

Time (min)	% A	% B	Flow
0	40	60	0.5 mL/min
25	40	60	0.5 mL/min
25.1	0	100	1 mL/min

Column temperature: 30°C

Injection volume: 2 µL (needle wash mode)

Detection: UV at 254 nm

Analysis time: 30.0 min

Retention time:

Impurity 1: 19.9min

MS source: API-ES

Polarity: positive

Gas temperature: 350°C

SIM ion: 312, 314 at 14 min

Quantitation of impurity 1 was performed by the following equation:

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

$$Impurity1(\%) = \frac{W''}{W} \times 100$$

Where:

A = the average peak area of impurity 1 in test item solution

A' = the average peak area of impurity 1 in standard solution

W = the mass of test item

W' = the mass of impurity 1 standard

P = the purity of impurity 1 standard

Z-Azoxistrobin (impurity 3) - HPLC/UV

System: Agilent 1200 HPLC system with DAD

Column: Eclipse Plus C8, 100 mm x 4.6 mm, 3.5 µm

Mobile phase A: water (0.1% H₃PO₄)

Mobile phase B: Acetonitrile

Gradient:

Time (min)	% A	% B
0	45	55
7.00	45	55
7.10	5	95

Flow: 0.5 mL/min

Column temperature: 35°C

Injection volume: 1 µL (needle wash mode)

Detection: UV at 210 nm

Analysis time: 16.0 min

Retention time: 8.9 min

Quantitation of impurity 3 was performed by the following equation:

$$W'' = \frac{A}{A'} \times W' \times P \times 0.01$$

$$Impurity3(\%) = \frac{W''}{W} \times 100$$

Where:

A = the average peak area of impurity 3 in test item solution

A' = the average peak area of impurity 3 in standard solution

W = the mass of test item

W' = the mass of impurity 3 standard

P = the purity of impurity 3 standard

Toluene (impurity 4) - GC-FID

System: Agilent 7890 GC with FID detector

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

Carrier gas: N₂

Flow program: 1.0 mL/min for 12 min, then 10 mL/min

Run time: 30.4 min

Oven temperature:

Initial: 80°C

Hold time: 12.0 min

Rate: 50°C/min
Final temperature: 250°C
Hold time: 15 min
Injector temperature: 200°C
Injection volume: 1 µL (split ratio 5:1)
Temperature FID detector: 250°C
Gases:
H₂: 60 mL/min
Air: 300 mL/min
N₂: 25 mL/min
Retention time: 11.6 min

Quantitation of impurity 4 was performed by the following equation:

$$W'' = \frac{A}{A'} \times W' \times P \times 0.01$$

$$Impurity4(\%) = \frac{W''}{W} \times 100$$

Where:

A = the average peak area of impurity 4 in test item solution

A' = the average peak area of impurity 4 in standard solution

W = the mass of test item

W' = the mass of impurity 4 standard

P = the purity of impurity 4 standard

Validation - Results and discussions

Table 5.2-3: Methods suitable for the determination of the relevant impurity Prothioconazole-desthio in plant protection product (PPP) CA3642

	Prothioconazole-desthio
Author(s), year	Wang, Q. (2022)
Principle of method	HPLC-MS
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	The linearity of detector response was demonstrated using injections of six concentrations (in duplicate) of reference standard in the approximate range of 0.04 to 50.8 mg/L (equivalent to 0.0004% w/w – 0.508% w/w prothioconazole-desthio in the test item). This range covers 80% to 120% of impurity level quantified in test item (precision) and also 80% to 120% of max. impurity level in product. y = 22526873.2178 x + 641699.1787 R = 0.9998
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability (precision) was determined from single determinations of five samples of CA3642. Mean content: 0.0031% w/w RSD: 3.23% RSDr: 6.39% Hr (Horrat value): 0.51 Intermediate precision: Another analyst, another day Mean content: 0.0032% w/w RSD: 6.26% RSDr: 6.36% Hr (Horrat value): 0.98
Accuracy n = 5 (% Recovery)	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.
Interference/ Specificity	Samples of blank formulation, reference item solution and test item solution were analysed. No additional analytical signals in the mean retention time of the impurity

	Prothioconazole-desthio
	prothioconazole-desthio were observed. Relevant chromatograms are provided.
LOQ	The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable accuracy and precision data is obtained, was determined to be 0.0005% w/w (max. impurity level 0.007 % w/w).
LOD	The limit of detection was calculated to be 0.00004% w/w (three times signal to noise ratio).
Comment	-

Table 5.2-4: Prothioconazole-desthio Accuracy data - Wang, Q. (2022)

Fortification Level (mg/L)			No of Determinations	Mean Recovery (%)	RSD (%)	Acceptable Recovery (%)
mg pure Prothioconazole-desthio in L acetonitrile	% w/w pure Prothioconazole-desthio in product CA3642	g Prothioconazole-desthio in L product				
0.0500	0.000500% w/w	0.0055	5	116.00	7.71	75 – 125*
1.5105	0.015105% w/w	0.166	5	100.23	0.75	75 – 125

* 70-130% according to SANCO 3030/99 rev. 5 as % w/w in test item is below 0.01%.

Table 5.2-5: Methods suitable for the determination of the relevant impurities Z-Azoxystrobin and Toluene in plant protection product (PPP) CA3642

	Z-Azoxystrobin	Toluene
Author(s), year	Wang, Q. (2022)	Wang, Q. (2022)
Principle of method	HPLC-DAD _(210nm)	GC-FID
Linearity (linear between mg/L) (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 1.98 to 180.6 mg/L (equivalent to 0.0020% w/w – 0.1806% w/w Z-azoxystrobin in the test item). This range covers 80% to 120% of impurity level quantified in test item (precision). $y = 12.9688x - 1.0381$ $R = 1.000$	The linearity of detector response was demonstrated using injections of five concentrations (in duplicate) of reference standard in the approximate range of 0.99 to 59.70 mg/L (equivalent to 0.0010% w/w – 0.0597% w/w toluene in the test item). This range covers 80% to 120% of impurity level quantified in test item (precision). $y = 5.2106x - 1.4644$ $R = 0.9999$
Precision – Repeatability Mean n = 5 (%RSD)	Repeatability (precision) was determined from single determinations of five samples of CA3642. Mean content: 0.0038% w/w RSD: 1.84% RSDr: 6.20% Hr (Horrat value): 0.30 Intermediate precision: Another analyst, another day Mean content: 0.0038% w/w RSD: 1.84% RSDr: 6.20% Hr (Horrat value): 0.30	Repeatability (precision) was determined from single determinations of five samples of CA3642. Mean content: 0.0064% w/w RSD: 1.09% RSDr: 5.73% Hr (Horrat value): 0.19 Intermediate precision: Another analyst, another day Mean content: 0.0063% w/w RSD: 2.06% RSDr: 5.75% Hr (Horrat value): 0.36
Accuracy n = 5 (% Recovery)	Recovery was determined using replicate injections of test item which had been fortified with known concentrations of Z-azoxystrobin at two fortification levels. The results are presented in Table 5.2-6 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 5.2-7 below.
Interference/ Specificity	Samples of blank formulation, reference item solution and test item solution were analysed.	Samples of blank formulation, reference item solution and test item solution were analysed.

	Z-Azoxystrobin	Toluene
	No additional analytical signals in the mean retention time of the impurity Z-azoxystrobin were observed. Relevant chromatograms are provided.	No additional analytical signals in the mean retention time of impurity toluene were observed in Test item solutions. Relevant chromatograms are provided.
LOQ	The limit of quantification (LOQ), defined as the lowest fortification level at which acceptable accuracy and precision data is obtained, was determined to be 0.00301% w/w (max. impurity level 0.367 % 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 0.00149% w/w (max. impurity level 0.10 % w/w).
LOD	The limit of detection was calculated to be 0.00012% w/w (three times signal to noise ratio).	The limit of detection was calculated to be 0.00037% w/w (three times signal to noise ratio).
Comment	-	-

Table 5.2-6: Z-Azoxystrobin Accuracy data - Wang, Q. (2022)

Fortification Level (mg/L)			No of Determinations	Mean Recovery (%)	RSD (%)	Acceptable Recovery (%)
mg pure Z-azoxystrobin in L acetonitrile	% w/w pure Z-azoxystrobin in product CA3642*	g Z-Azoxystrobin in L product				
3.0073	0.00301%/w/w	0.0330	5	99.62	2.92	75 – 125*
50.1221	0.0501% w/w	0.5515	5	98.61	0.40	75 - 125

* 70-130% according to SANCO 3030/99 rev.5 as % w/w in test item is below 0.01%.

Table 5.2-7: Toluene Accuracy data - Wang, Q. (2022)

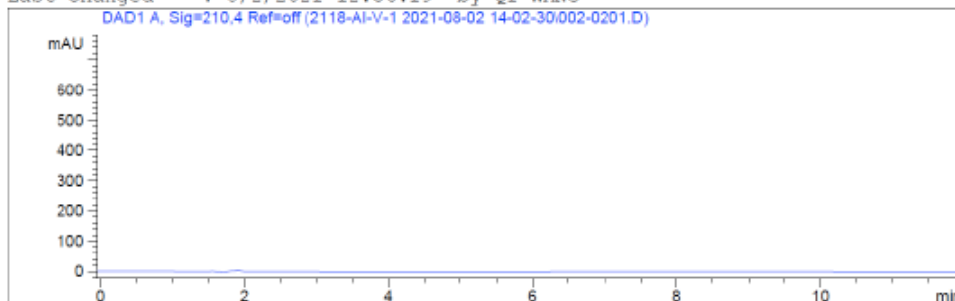
Fortification Level (mg/L)			No of Determinations	Mean Recovery (%)	RSD (%)	Acceptable Recovery (%)
mg pure Toluene in L acetonitrile	% w/w pure Toluene in product CA3642	g Toluene in L product				
1.4880	0.00149% w/w	0.0164	5	94.09	2.63	75 – 125
9.9201	0.00992% w/w	0.1092	5	85.28	3.78	75 – 125

Chromatogram obtained from solution of Blank formulation

Test Method: ABCTM-2021-018-02

Data File E:\GLP\2021\ABC-2021-018\2118-AI-V-1 2021-08-02 14-02-30\002-0201.D
Sample Name: 2118-T2-V-001

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    2
Acq. Instrument : Instrument 2                  Location  : Vial 2
Injection Date  : 8/2/2021 14:18:14             Inj       :    1
                                           Inj Volume: 3 µl
Sequence File   : E:\GLP\2021\ABC-2021-018\2118-AI-V-1 2021-08-02 14-02-30\2118-AI-V-1.S
Method          : E:\GLP\2021\ABC-2021-018\2118-AI-V-1 2021-08-02 14-02-30\ABCTM-2021-018-02.M
Last changed    : 8/2/2021 12:56:19 by QI WANG
DAD1 A, Sig=210.4 Ref=off (2118-AI-V-1 2021-08-02 14-02-30\002-0201.D)
```



Area Percent Report

```
Sorted By      : Signal
Multiplier     : 1.0000
Dilution       : 1.0000
Use Multiplier & Dilution Factor with ISTDs
```

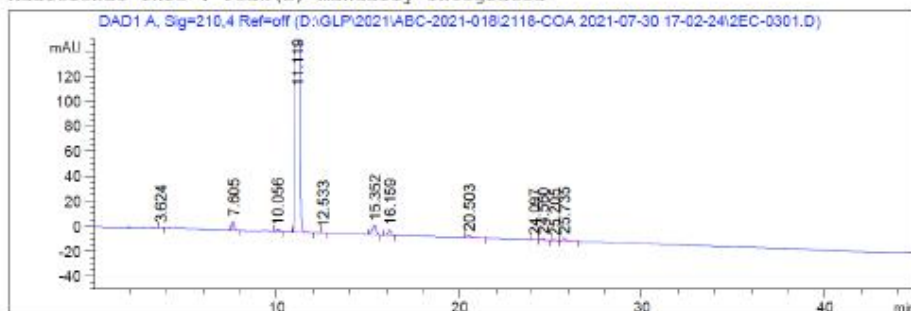
No peaks found

Chromatogram obtained from a Reference Item solution:

Test Method: ABCTM-2021-018-03

Data File D:\GLP\2021\ABC-2021-018\2118-COA 2021-07-30 17-02-24\2EC-0301.D
Sample Name: EPP/RH1928.10

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    3
Acq. Instrument : ABC-GLP-044                  Location  : P2-E-03
Injection Date  : 30/07/2021 18:55:55           Inj       :    1
                                           Inj Volume: 0.200 µl
Different Inj Volume from Sample Entry! Actual Inj Volume : 2.000 µl
Acq. Method     : D:\GLP\2021\ABC-2021-018\2118-COA 2021-07-30 17-02-24\ABCTM-2021-018-01.M
Last changed    : 30/07/2021 14:54:48 by QI WANG
Analysis Method : D:\GLP\2021\ABC-2021-018\2118-COA 2021-07-30 17-02-24\ABCTM-2021-018-01.M (
Sequence Method)
Last changed    : 31/07/2021 08:42:02 by QI WANG
(modified after loading)
Additional Info  : Peak(s) manually integrated
```

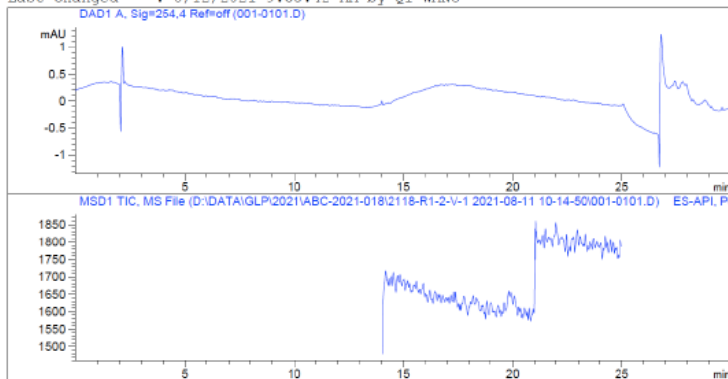


Chromatogram obtained from a solution of blank:

Test Method: ABCTM-2021-018-03

Data File D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\001-0101.D
Sample Name: 2118-B1-V-004

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    1
Acq. Instrument : Instrument 1                  Location  : Vial 1
Injection Date  : 8/11/2021 10:30:09 AM         Inj       :    1
                                                Inj Volume: 2.0 µl
Sequence File   : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\2118-R1-2-V-1.
S
Acq. Method     : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-
018-03.M
Last changed    : 8/11/2021 10:14:01 AM by QI WANG
Analysis Method : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-
018-03.M
Last changed    : 8/12/2021 9:55:42 AM by QI WANG
=====
```



Area Percent Report

```
=====
Sorted By      :      Signal
Multiplier:    :      1.0000
Dilution:      :      1.0000
Use Multiplier & Dilution Factor with ISTDs

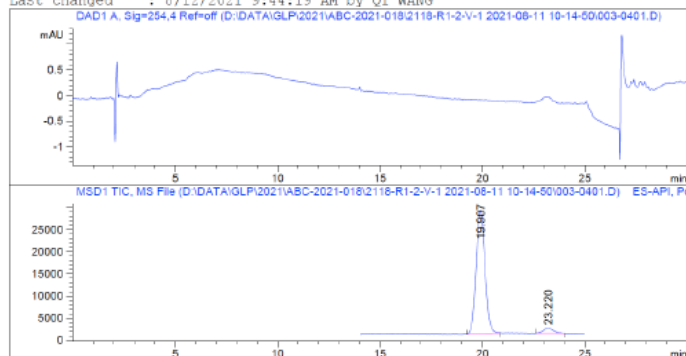
No peaks found
=====
```

Chromatogram obtained from a Reference Items solution (0.3 mg/L Impurity I + 0.5 mg/L Impurity II)

Test Method: ABCTM-2021-018-03

Data File D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\003-0401.D
Sample Name: 2118-R1-V-101

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    4
Acq. Instrument : Instrument 1                  Location  : Vial 3
Injection Date  : 8/11/2021 12:19:42 PM         Inj       :    1
                                                Inj Volume: 2.0 µl
Acq. Method     : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-
018-03.M
Last changed    : 8/11/2021 10:14:01 AM by QI WANG
Analysis Method : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-
018-03.M
Last changed    : 8/12/2021 9:44:19 AM by QI WANG
=====
```

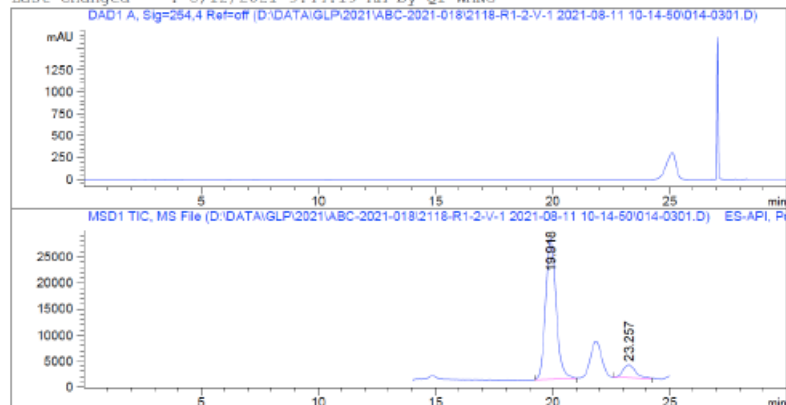


Chromatogram obtained from a Test Item solution (Test Item solution: 10 g/L):

Test Method: ABCTM-2021-018-03

Data File D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\014-0301.D
Sample Name: 2118-T1-V-057

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    3
Acq. Instrument : Instrument 1                  Location  : Vial 14
Injection Date  : 8/11/2021 11:43:11 AM         Inj       :    1
                                           Inj Volume: 2.0 µl
Acq. Method     : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-018-03.M
Last changed    : 8/11/2021 10:14:01 AM by QI WANG
Analysis Method : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-018-03.M
Last changed    : 8/12/2021 9:44:19 AM by QI WANG
=====
```



Area Percent Report

```
=====
Sorted By      : Signal
Calib. Data Modified : Thursday, August 12, 2021 9:46:25 AM
Multiplier:    : 1.0000
Dilution:      : 1.0000
Use Multiplier & Dilution Factor with ISTDs
=====
```

Signal 1: DAD1 A, Sig=254,4 Ref=off

Signal 2: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Area %	Name
1	19.918	BBA	0.4636	8.38331e5	90.2000	Impurity I
2	23.257	BV	0.4464	9.10825e4	9.8000	Impurity II

Totals : 9.29413e5

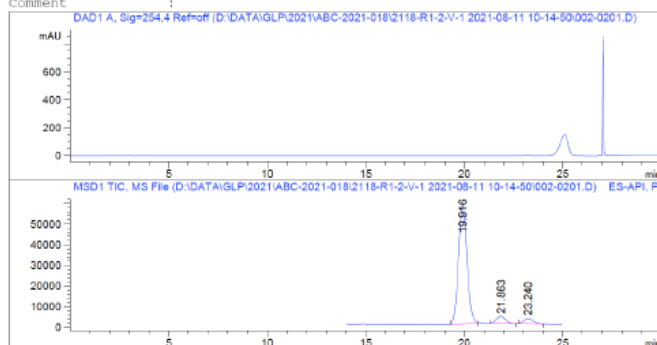
Spectra of performance for Impurity I and II obtained from mixed solution of Impurity I and II standards and Test Item Prothioconazole 150 g/L+ Azoxystrobin 150 g/L S.C.:

Test Method: ABCTM-2021-018-03

Data File D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\002-0201.D
Sample Name: 2118-T1-V-056

```
=====
Acq. Operator   : QI WANG                      Seq. Line :    2
Acq. Instrument : Instrument 1                  Location  : Vial 2
Injection Date  : 8/11/2021 11:06:40 AM         Inj       :    1
                                           Inj Volume: 2.0 µl
Acq. Method     : D:\DATA\GLP\2021\ABC-2021-018\2118-R1-2-V-1 2021-08-11 10-14-50\ABCTM-2021-018-03.M
Last changed    : 8/11/2021 10:14:01 AM by QI WANG
Analysis Method : D:\DATA\GLP\2021\METHOD\ABCTM-2021-018-03.M
Last changed    : 8/12/2021 12:38:13 PM by QI WANG
                  (modified after loading)
=====
```

```
Column Description : Eclipse Plus C8
Product#          : 959961-906                Batch#: B16087
Serial#           : USUTR01693
Diameter          : 4.6 mm                    Length : 100.0 mm
Particle size     : 3.5 µm                    Void volume: 60.0 %
Maximum Pressure  : 400 bar                    Maximum pH :    9
Maximum Temperature: 60 °C
Comment          :
```



Area Percent Report with Performance

```
Multiplier:      : 1.0000
Dilution:        : 1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: DAD1 A, Sig=254.4 Ref=off

Signal 2: MSD1 TIC, MS File

RetTime [min]	k'	Area	Height	Symm.	Width [min]	Plates	Resol	Select ivity
19.916	8.99	1.72880e6	5.74769e4	0.90	0.4635	10229	-	-
21.863	9.96	1.07523e5	3392.41968	0.99	0.5012	10542	2.37	1.11

Conclusion

The validation of the methods for analysis of relevant impurities prothioconazole-desthio, Z-azoxystrobin and toluene in CA3642 have 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 methods are acceptable for the quantification of relevant impurities prothioconazole-desthio, Z-azoxystrobin and toluene in CA3642. The LOQs of the methods are respectively 0.0005% w/w for prothioconazole-desthio, 0.00301% w/w for Z-azoxystrobin and 0.00149% w/w for toluene.

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

CA3642 does not contain any (eco)toxicologically or environmental relevant formulants.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

CIPAC methods are only applicable to single active substances, consequently, as CA3642 contains a mixture of active substances, there are no directly applicable methods to the product. Methods relevant to the individual active substances are outlined below.

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.

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

Azoxystrobin [571] Handbook M:

Outline of method

The sample is dissolved in acetone containing an internal standard and the azoxystrobin content determined by capillary gas chromatography.

The method is usable for TC, WG 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 and azoxystrobin for the generation of pre-authorization data is given in the following tables. For the detailed evaluation of studies it is referred to Appendix 2.

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

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 prothioconazole and prothioconazole-desthio (wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber)	LC-MS/MS	KCP 5.1.2/01 Winter O. and Giesler W., 2017, report No. S16-04434 (NUD-1601V)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. <u>12</u>	
	Primary	0.01 mg/kg prothioconazole- α -hydroxy-desthio, prothioconazole3-, -4-, -5- and -6-hydroxy- desthio, prothioconazole-desthio-3- glucoside, prothioconazole-desthio-4- glucoside, prothioconazole-desthio-6- glucoside in wheat (whole plant, grain and straw) and oilseed rape (seeds)	LC-MS/MS	KCP 5.1.2/02 Winter O. & Nachtigall S, 2020, report No. S16-04435 (NUD-1602V)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. <u>12</u>	
	Primary	0.01 mg/kg 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	LC-DMS/MS/MS	KCP 5.1.2/03 Schernikau N. and Suaza Colorado C., 2016, report No. S15-03542 (GAB- 1537V)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. <u>12</u>	
	Primary	0.01 mg/kg 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	LC-DMS/MS/MS	KCP 5.1.2/04 Class, T., 2011, report No. P 2383G, M- 420638-01-1
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. <u>12</u>	
	Primary	0.01mg/kg (oilseed rape)	LC-MS/MS	KCP 5.1.2/05 North L., 2021, Report No. S19-01269
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. <u>12</u>	
	Primary	0.01 mg/kg (oilseed rape)	LC-MS/MS	KCP 5.1.2/06 North L., 2021, Report No. S20-01046
	Confirmatory		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. 2	
	Primary	0.01 mg/kg (wheat)	LC-MS/MS	KCP 5.1.2/07 North L., 2020 Report No. S19-01268
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.01 mg/kg (wheat grain, potato tuber, tomato fruit, rape seed, orange fruit)	HPLC-MS/MS	KCP 5.1.2/08 Freitag T., 2006 Report No. M-267072-01-1, New study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.01 mg/kg (orange fruit, bean seed, rape seed, cereal grain, strawberry, barley green material, wheat straw)	HPLC-MS/MS	KCP 5.1.2/10 Glaubitz J. and Hennes M, 2016, Report No. M-513336-02-1, New study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.01 mg/kg (citrus fruit, pea, green sead, rape seed, wheat grain, corn green material)	HPLC-MS/MS	KCP 5.1.2/11 Brumhard B. and Stuke S, 2016, Report No. M-283439-04-1, New study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Please refer also to post authorisation methods.			
Animal products, food of animal origin,... (Residues)	Primary	0.01 mg/kg (nectar and pollen) 66.666 mg/L of spray solutions	HPLC-MS/MS	KCP 5.1.2/12 Bocksch S., 2023, Report No. S21-00461, New study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Please refer also to post authorisation methods.			

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
Soil, water, sediment,... (Environmental fate)	Not applicable.			
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	50% aqueous sucrose solution: 2.08 mg prothioconazole/kg	HPLC-MS/MS	KCP 5.1.2/13 Gimeno I., 2022, report No. S21-04081, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 1 2	
	Primary	Deionised water: 12.5 mg prothioconazole/L	HPLC-MS/MS	KCP 5.1.2/14 Gimeno I., 2022, report No. S21-04082, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 1 2	
	Primary	50% aqueous sucrose solution: 27.7 mg prothioconazole/L 0.1% Triton: 277 mg prothioconazole/L	HPLC-MS/MS	KCP 5.1.2/15 Gimeno I., 2022, report No. S21-04083, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 1 2	
	Primary	Tap water: 216 mg prothioconazole/L	HPLC-MS	KCP 5.1.2/16 Huerta F., 2022, report No. S21-04084, New Study
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 1 2	
	Primary	Test water: 1.36 µg prothioconazole/L	HPLC-MS/MS	KCP 5.1.2/17, Wenzel B., Marini P., 202 3 , report No. 20210195, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk	

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
			assessment according to SANTE/2020/12830, Rev. 2	
	Primary	Test water: 2.84 µg prothioconazole/L	HPLC-MS/MS	KCP 5.1.2/18, Dupont A., 2022, report No. 20210196, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	Test water: 0.0617 µg prothioconazole/L	HPLC-MS/MS	KCP 5.1.2/19 Dupont A., 2022, report No. 20210197, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
Water, buffer solutions,... (Properties)	Primary	0.5 mg prothioconazole/L (acetonitrile rinse and tank mix solutions)	HPLC-UV	KCP 5.1.2/23 Calvert A., 2023, report No. 23/1610
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	

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

Component of residue definition: Azoxystrobin				
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 azoxystrobin and azoxystrobin-Z-isomer in wheat (grain, straw and processed fractions), barley (grain, straw and processed fractions), oilseed rape (grain, cake and oil)	LC-MS/MS	Refer to post authorisation methods (KCP 5.2/01 Kawa-Miszczak L., 2011, report No. PBBZ-2011/07/DPL (ChR-10-8231, New study))
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
Animal products, food of animal origin,... (Residues)	Primary	0.01 mg/kg azoxystrobin and azoxystrobin-Z-isomer in honey	LC-MS/MS	KCP 5.1.2/20 Bocksch S., 2008, Report No. T011298-06-REG, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Primary	0.005 mg/kg azoxystrobin in oilseed rape pollen and artificial nectar	LC-MS/MS	KCP 5.1.2/21 Lebrun F., 2019, Report No. 349-2018, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.01 mg/kg azoxystrobin and azoxystrobin-Z-isomer in honey	LC-MS/MS	KCP 5.1.2/22 Appeltauer A, 2022, Report No. S21-01128, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.001 mg/kg azoxystrobin and azoxystrobin-Z-isomer in milk	GC-NPD	Ryan J. and Sapiets A., 1996, Report No. RJ1809B
	Confirmatory	0.01 mg/kg Azoxystrobin and azoxystrobin-Z-isomer for animal tissue and eggs	Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	EU agreed (evaluated and accepted in the RAR (2009))
	Primary	0.01 mg/kg azoxystrobin and azoxystrobin-Z-isomer in bovine whole milk, poultry's eggs, bovine fat, bovine muscle meat and bovine liver	LC-MS/MS	Refer to post-authorisation methods (KCP 5.2/07 Siekmann D., 2017, Report No.S17-01577, New study)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.02 mg/kg azoxystrobin (nectar and pollen) 66.666 mg/L azoxystrobin in spray solutions	HPLC-MS/MS	KCP 5.1.2/12 Bocksch S., 2023, Report No. S21-00461, New study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
Soil, water, sediment,... (Environmental fate)	Primary	0.02 mg/kg azoxystrobin, azoxystrobin-Z-isomer and R234886 metabolite in soil 0.01 mg/kg for R401553 and R402173 metabolites in soil	LC-MS/MS	Refer to post-authorisation methods Johnson R.I., 2000, Report reference RAM 269/03 EU agreed (evaluated and accepted in the RAR (2009))
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to	

Component of residue definition: Azoxystrobin				
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. 2	
	Primary	0.1 µg/L for azoxystrobin in surface water, ground water and drinking water	GC-MSD	Refer to post-authorisation methods Robinson N.J., 2000, Report reference RAM 358/01 EU agreed (evaluated and accepted in the RAR (2009))
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
Soil, water,... (Efficacy)	Not applicable			
Feed, body fluids,... (Toxicology) Body fluids, air,... (Exposure)	Primary	0.003 mg/m³ azoxystrobin in air (ambient and 35°C, 80% RH)	GC-MSD	Refer to post-authorisation methods Crawford N., 2001, Report No. TMJ4658B EU agreed (evaluated and accepted in the RAR (2009))
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.05 µg/mL azoxystrobin and R234886 metabolite in human and dog plasma	HPLC-UV	Hall M.G., 1999, Report No. CTL/R/1401 EU agreed (evaluated and accepted in the RAR (2009))
	Confirmatory		LC-MS	
	Primary	0.05 µg/mL azoxystrobin and R234886 metabolite in human plasma and urine	LC-MS/MS	Refer to post authorisation methods (KCP 5.2/17 Amic S., 2011, Report No. S11-02193, New study)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.05 mg/L azoxystrobin and azoxystrobin-Z-isomer in human urine	LC-MS/MS	Refer to post authorisation methods (KCP 5.2/16 Siekmann D., 2017, Report No. S17-01576, New study)
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
Soil, water,...	Primary	2.11 mg azoxystrobin/kg	HPLC-MS/MS	KCP 5.1.2/13 Gimeno I., 2022, report No. S21-

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
(Ecotoxicology)	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	04081, New Study
	Primary	12.7 mg azoxystrobin/L	HPLC-MS/MS	KCP 5.1.2/14, Gimeno I., 2022, report No. S21-04082, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	50% aqueous sucrose solution: 28.1 mg azoxystrobin/L 0.1% Triton: 281 mg azoxystrobin/L	HPLC-MS/MS	KCP 5.1.2/15 Gimeno I., 2022, report No S21-04083, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	219 mg azoxystrobin/L	HPLC-MS	KCP 5.1.2/16 Huerta F., 2022, report No S21-04084, New Study
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	1.38 µg azoxystrobin/L	HPLC-MS/MS	KCP 5.1.2/17 Wenzel B., Marini P., 2023, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	2.89 µg azoxystrobin/L	HPLC-MS/MS	KCP 5.1.2/18 Dupont A., 2022, report No. 20210196, New Study
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	
	Primary	0.0627 µg azoxystrobin/L	HPLC-MS/MS	KCP 5.1.2/19 Dupont A., 2022, report No.

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	Confirmatory		Self confirmatory MS/MS. Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	20210197, New Study
Water, buffer solutions,... (Properties)	Primary	0.5 mg azoxystrobin/L (acetonitrile rinse and tank mix solutions)	HPLC-UV	KCP 5.1.2/23 Calvert A., 2024, report No. 23/1610
	Confirmatory		Not required for methods for risk assessment according to SANTE/2020/12830, Rev. 2	

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 substances 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 and azoxystrobin (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.

Matrix	Residue definition (prothioconazole)		
	EFSA Conclusion Scientific Report (2007) 106	EFSA Reasoned Opinion Journal 2014;12(5):3689	COMMISSION REGULATION (EU) 2010/552 2024/1318
Plants	Prothioconazole-desthio. (JAU 6476-desthio)	/	Prothioconazole: prothioconazole-desthio (sum of isomers) (F)
Animals	Sum of prothioconazole-desthio and its glucuronide conjugate, expressed as prothioconazole-desthio (JAU 4676-desthio)	prothioconazole-desthio (sum of isomers)	Prothioconazole: prothioconazole-desthio (sum of isomers) (F)
Soil	prothioconazole, prothioconazole-desthio (M04)	/	/
Water	prothioconazole and prothioconazole-desthio (M04)	/	/
Ground, surface			
Sediment	Prothioconazole	/	/
Air	prothioconazole and prothioconazole-desthio (M04)	/	/

Matrix	Residue definition (azoxystrobin)	
	EFSA Journal 2010; 8(4):1542	COMMISSION REGULATION (EU) 2023/129 2024/1078 2024/2633
Plants	Azoxystrobin and Azoxystrobin-Z-isomer	Azoxystrobin
Animals	Azoxystrobin and Azoxystrobin-Z-isomer	Azoxystrobin
Soil	Azoxystrobin	Azoxystrobin
Water	Azoxystrobin	Azoxystrobin
Ground, surface		
Sediment	Azoxystrobin	Azoxystrobin
Air	Azoxystrobin	Azoxystrobin

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content	Prothioconazole-desthio (sum of isomers)	LOQ: 0.01 mg/kg*	Reg. (EU) 2019/552 2024/1318
Plant, high water content		LOQ: 0.01 mg/kg*	Reg. (EU) 2019/552 2024/1318
Cereals, grains (Plants, 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 2024/1318
Oilseed, seeds (Plants, high oil content)		Gold of pleasure MRL: 0.04 mg/kg Linseed MRL: 0.09 mg/kg Mustard MRL: 0.09 mg/kg Poppy MRL: 0.09 mg/kg Rapeseed MRL: 0.15 mg/kg Sunflower MRL: 0.2 mg/kg	Reg. (EU) 2019/552 2024/1318
Plants, difficult matrices (hops, spices, tea)		LOQ: 0.05 mg/kg*	Reg. (EU) 2019/552 2024/1318
Plant, High oil content		LOQ: 0.02 mg/kg*	Reg. (EU) 2019/552 2024/1318
Ruminants, Muscle	Prothioconazole-desthio (sum of isomers)	MRL: 0.01 mg/kg	Reg. (EU) 2019/552 2024/1318
Ruminants, Fat		MRL: 0.02 mg/kg	
Ruminants, Liver, kidney		MRL: 0.5 mg/kg	
Milk		LOQ: 0.01* mg/kg	
Poultry, Muscle		LOQ: 0.01* mg/kg	
Poultry, Fat		LOQ: 0.01* mg/kg	
Poultry, Liver, kidney		MRL: 0.1 mg/kg	
Eggs		LOQ: 0.01* mg/kg	
Soil (Ecotoxicology)	Prothioconazole and 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 <i>O. mykiss</i> (EFSA Journal (2007) 106, 1-98)
Air	Prothioconazole and prothioconazole-desthio	0.015 mg/m ³ prothioconazole 0.0006 mg/m ³ prothioconazole- desthio	AOEL prothioconazole = 0.2 mg/kg bw/d C = 60 µg/m ³ (0.06 mg/m ³) AOEL prothioconazole-desthio = 0.01 mg/kg bw/d = 3 µg/m ³ (0.003 mg/m ³) Conversions according to SANTE/2020/12830, Rev.-2 (EFSA Journal (2007) 106, 1-98)
Tissue (meat or liver)	N/A	0.01 mg/kg	SANTE/2020/12830, Rev.-2
Body fluids		0.01 mg/kg	SANTE/2020/12830, Rev.-2

* Indicates lower limit of analytical determination

Table 5.3-2: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required (azoxystrobin)

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high acid content	Azoxystrobin	LOQ/MRL: 0.01* mg/kg Citrus fruits MRL: 15 mg/kg Grapes MRL: 3 mg/kg Cane fruits MRL: 5 mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Plant, high water content		LOQ/MRL: 0.01* mg/kg Apple LOQ: 0.01 mg/kg Pears LOQ: 0.01 mg/kg Apricots MRL: 2 mg/kg Peaches MRL: 2mg/kg Mangoes MRL: 4 mg/kg Papayas MRL: 0.3 4 mg/kg Tomatoes MRL: 3 mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Cereals, grains (Plants, high starch content/dry commodities)		LOQ/MRL: 0.01* mg/kg MRL: 1.5 mg/kg Oat MRLs: 1.5 mg/kg Rye MRL: 0.5 mg/kg Wheat MRL: 0.5 mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Oilseed, seeds (Plants, high oil content)		LOQ/MRL: 0.01* mg/kg Gold of pleasure MRL: 0.5 mg/kg Linseed MRL: 0.4 mg/kg Mustard MRL: 0.5 mg/kg Poppy MRL: 0.5 mg/kg Rapeseed MRL: 0.7 mg/kg Sunflower MRL: 0.5 mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Plant, difficult matrices (hops, spices, tea)		LOQ/MRL: 0.05* mg/kg Seed spices MRL: 0.3 mg/kg Tea LOD: 0.05 mg/kg Hops MRL: 30 40 mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Ruminants, Muscle	All animals: Azoxystrobin	LOQ: 0.01* mg/kg	Reg. (EU) 2023/129 2024/1078 2024/2633
Ruminants, Fat		MRL: 0.05 mg/kg	
Ruminants, Liver, kidney		MRL: 0.07 mg/kg	
Milk		LOQ: 0.01* mg/kg	
Poultry, Muscle		LOQ: 0.01* mg/kg	
Poultry, Fat		LOQ: 0.01* mg/kg	
Poultry, Liver, kidney		LOQ: 0.01* mg/kg	
Eggs		LOQ: 0.01* mg/kg	
Honey		LOQ: 0.05* mg/kg	
Soil (Ecotoxicology)	Azoxystrobin	No limit proposed (LOQ 0.02 mg/kg)	EFSA Journal 2010; 8(4):1542
Drinking water (Human toxicology)	Azoxystrobin	0.1 µg/L	General limit for drinking water
Surface water (Ecotoxicology)	Azoxystrobin	No limit proposed (LOQ 0.1 µg/L)	EFSA Journal 2010; 8(4):1542
Air	Azoxystrobin	No limit proposed (LOQ 3 µg/m ³)	EFSA Journal 2010; 8(4):1542
Tissue (meat or liver)	Azoxystrobin	0.01 mg/kg	SANTE/2020/12830, Rev. 1 2
Body fluids		0.01 mg/L	SANTE/2020/12830, Rev. 1 2

* Indicates lower limit of analytical determination

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 and azoxystrobin in plant matrices is given in the following tables. For the detailed evaluation of studies it is referred to Appendix 2.

Table 5.3-3: 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 Report No.: 00086/M033
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.02 mg/kg (tomato)	GC-MS	Class, 2001 / EU agreed Report No.: P/B 484 G
	Primary	0.01 mg/kg (cucumber)	LC-MS/MS	KCP 5.1.2/01 Winter O., Giesler W., 2017, report no.: S16-04434
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.01 mg/kg (cucumber)	LC-MS/MS	KCP 5.1.2/01a Heinz N., 2024 report no.: S23-106298
High acid content	Primary	0.02 mg/kg (orange)	GC-MS	Weeren, P., 2000 / EU agreed Report No.: 00086/M033
	Confirmatory		Self-confirmatory, two mass transitions	
	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.		
	Primary	0.01 mg/kg (grapes)	LC-MS/MS	KCP 5.1.2/01 Winter O., Giesler W., 2017, report no.: S16-04434
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.01 mg/kg (grapes)	LC-MS/MS	KCP 5.1.2/01a Heinz N., 2024 report no.: S23-106298
High oil content	Primary	0.02 mg/kg (rape seed)	GC-MS	Weeren, P., 2000 / EU agreed Report No.: 00086/M033
	Confirmatory		Self-confirmatory, two mass transitions	
	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.		
	Primary	0.01 mg/kg (oilseed rape seeds)	LC-MS/MS	KCP 5.1.2/01 Winter O., Giesler W., 2017, report no.: S16-04434
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.01 mg/kg (oilseed rape seeds)	LC-MS/MS	KCP 5.1.2/01a Heinz N., 2024 report no.: S23-106298
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 Report No.: 00086/M033
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.02 mg/kg (wheat grain)	GC-MS	Class, 2001 / EU agreed Report No.: P/B 484 G

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 (wheat grain, beans (dry))	LC-MS/MS	KCP 5.1.2/01 Winter O., Giesler W., 2017, report no.: S16-04434
	Confirmatory		Self-confirmatory, two mass transitions	
	ILV	0.01 mg/kg (wheat grain, beans (dry))	LC-MS/MS	KCP 5.1.2/01a Heinz N., 2024 report no.: S23-106298
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.

zRMS comments:

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

The applicant provided sufficiently validated analytical method of Winter O., Giesler W. (2017, S16-04434) and the ILV of S16-04434 (Heinz N., 2024, S23-106298) for the determination of relevant residues of prothioconazole and prothioconazole-desthio in/on matrices of plant origin by HPLC-MS/MS with LOQ of 0.01 mg/kg. The methods are acceptable. The details of the evaluation of new and additional studies are referred in Appendix 2.

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

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg (cabbage and wheat forage)	LC-MS/MS	Lister, N., 1999 / EU agreed Report No. RJ2770B
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (Lettuce, kale and potato)	LC-MS/MS	Kang, J., 2003 / EU agreed Report No. CEMR-1708 v3
	Primary	0.01 mg/kg (beer and cabbage)	LC-MS/MS	Chaggar, S., 2004 / EU agreed Report No. RJ3552B
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (Cabbage and sugar beet root)	LC-MS/MS	Croucher, A., 2002 / EU agreed Report No. 1983/029-D2419
	Primary	0.01 mg/kg (green beans matrices, rotational crops matrices)	LC-MS/MS	KCP 5.2/01 Kawa-Miszczak L., 2011, Report No. PBBZ-2011/07/DPL, New study
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (wheat (whole plant))	LC-MS/MS	KCP 5.2/02 Lefresne S., 2011, Report No. NUFARM/AZO/11.01, New study
High acid content	Primary	0.01 mg/kg	LC-MS/MS	Chaggar, S., 2004 / EU agreed

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
		(mandarin)		Report No. RJ3552B
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	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	0.01 mg/kg (sunflower seed)	LC-MS/MS	Chaggar, S., 2004 / EU agreed Report No. RJ3552B
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	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.		
	Primary	0.01 mg/kg (oilseed rape matrices)	LC-MS/MS	KCP 5.2/01 Kawa-Miszcza L., 2011, Report No. PBBZ-2011/07/DPL, New study
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (oilseed rape (grain))	LC-MS/MS	KCP 5.2/02 Lefresne S., 2011, Report No. NUFARM/AZO/11.01, New study
High protein/high starch content (dry)	Primary	0.01 mg/kg (barley grain and wheat straw)	LC-MS/MS	Lister, N., 1999 / EU agreed Report No. RJ2770B
	Primary	0.01 mg/kg (wheat straw, wheat flour)	LC-MS/MS	Chaggar, S., 2004 / EU agreed Report No. RJ3552B
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (maize kernel, maize plant)	LC-MS/MS	Croucher, A., 2002 / EU agreed Report No. 1983/029-D2419
	Primary	0.01 mg/kg (winter wheat matrices, Summer barley matrices, oilseed rape matrices, green beans matrices, rotational crops matrices)	LC-MS/MS	KCP 5.2/01 Kawa-Miszcza L., 2011, Report No. PBBZ-2011/07/DPL, New study
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		
	ILV	0.01 mg/kg (wheat (grain, whole plant), oilseed rape (grain))	LC-MS/MS	KCP 5.2/02 Lefresne S., 2011, Report No. NUFARM/AZO/11.01, New study
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 5.3-5: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	<p>According to SANTE 2017/10632 Rev. 4, 23 February 2022, 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.</p> <p>Prothioconazole is currently under renewal process which is not finalised yet – consequently, this should not be required.</p> <p>Azoxystrobin is currently under a second renewal process which is not finalised yet – consequently, this should not be required.</p>

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 5.3-6: Validated methods for food and feed of animal origin (if appropriate) - Prothioconazole

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	Heinemann, 2001c / EU agreed Report No.: 00655/M001
	ILV	0.004 mg/kg	HPLC-MS/MS	Dubey, 2001 / EU agreed Report No.: A-14-01-01
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2001b / EU agreed Report No.: 00655
	ILV	0.01 mg/kg	HPLC-MS/MS	Dubey, 2001 / EU agreed Report No.: A-14-01-01
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	Heinemann, 2001b / EU agreed Report No.: 00655
	ILV	0.01 mg/kg	HPLC-MS/MS	Dubey, 2001 / EU agreed Report No.: A-14-01-01
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		EU agreed
Eggs	Primary	0.01 mg/kg	HPLC-MS/MS	KCP 5.2/03 Schulte, G.; Oel, D., 2014, Report n°M-279725-03-1, New study
	ILV	0.01 mg/kg	HPLC-MS/MS	KCP 5.2/04 Bacher, R, 2006, Report n°M-279818-01-1, New study
	Confirmatory	LC-MS/MS highly specific - no need for confirmatory methods.		-
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	KCP 5.2/05 Kalathoor, R., 2021, S20-09747, New study
	ILV		HPLC-MS/MS	KCP 5.2/06 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 5.3-7: Validated methods for food and feed of animal origin (if appropriate) - Azoxystrobin

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/07 Sieckmann D., 2017, S17-01577, New study

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
	ILV		LC-MS/MS	KCP 5.2/08 Meryer M., 2017, S17-02332, New study
	Confirmatory	0.01 mg/kg	Self confirmatory (MS/MS)	See primary method and ILV
Muscle	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/07 Sieckmann D., 2017, S17-01577, New study
	ILV		LC-MS/MS	KCP 5.2/08 Meryer M., 2017, S17-02332, New study
	Confirmatory	0.01 mg/kg	Self confirmatory (MS/MS)	See primary method and ILV
Kidney, liver	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/07 Sieckmann D., 2017, S17-01577, New study
	ILV		According to SANTE/2020/12830, if the primary method is identical for all animal matrices, it is sufficient to perform the ILV with at least two of these matrices. This is applicable here.	
	Confirmatory	0.01 mg/kg	Self confirmatory (MS/MS)	See primary method and ILV
Fat	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/07 Sieckmann D., 2017, S17-01577, New study
	ILV		LC-MS/MS	KCP 5.2/08 Meryer M., 2017, S17-02332, New study
	Confirmatory	0.01 mg/kg	Self confirmatory (MS/MS)	See primary method and ILV
Eggs	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/07 Sieckmann D., 2017, S17-01577, New study
	ILV		According to SANTE/2020/12830, if the primary method is identical for all animal matrices, it is sufficient to perform the ILV with at least two of these matrices. This is applicable here.	
	Confirmatory		Self confirmatory (MS/MS)	See primary method and ILV
Honey	Primary	0.01 mg/kg	LC-MS/MS	KCP 5.2/09 Harper, H. 2022, Report No.8485926, New study
	ILV		LC-MS/MS	KCP 5.2/10 Homazaya, N.2022, Report No.20210438, New study
	Confirmatory		Self confirmatory (MS/MS)	See primary method and ILV

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 5.3-8: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	Please refer to justification in Table 5.3-5.

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

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

Table 5.3-9: Validated methods for soil (if appropriate) - Prothioconazole

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 Report No. 00610
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 Report No.: 00086/M038
Confirmatory		-	

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

Table 5.3-10: Validated methods for soil (if appropriate) - Azoxystrobin

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.02 mg/kg (azoxystrobin, z- isomer azoxystrobin, metabolites R234886, R410553, R402173)	HPLC-MS/MS	Johnson, R.I., 2000 / EU agreed Report No. 269/03
Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary	
Primary	0.01 mg/kg (azoxystrobin, R230310, R234886, R401553, R402173)	HPLC-MS/MS	KCP 5.2/11, Amic S., 2011, Report No. S11-02190 / New study
Confirmatory		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 soil please refer to Appendix 2.

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

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

Table 5.3-11: Validated methods for water (if appropriate) – Prothioconazole

Component of residue definition: Prothioconazole and prothioconazole-desthio				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole- desthio)	HPLC-MS/MS	Sommer, 2001b / EU agreed Report No.: 00684
	Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	

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
	Primary	0.05 µg/L (prothioconazole and prothioconazole-desthio)	HPLC-MS/MS	KCP 5.2/12 Krebber, R.; Sandau, C., 2015, Report No. M-526061-01-1, New study
	Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	
Surface water	Primary	0.1 µg/L (prothioconazole) 0.05 µg/L (prothioconazole-desthio)	HPLC-MS/MS	Sommer, 2001b / EU agreed Report No.: 00684
	Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	
Surface water	Primary	0.05 µg/L (prothioconazole and prothioconazole-desthio)	HPLC-MS/MS	KCP 5.2/12 Krebber, R.; Sandau, C., 2015, Report No. M-526061-01-1, New study
	Confirmatory		Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.	
	ILV	0.05 µg/L (prothioconazole and prothioconazole-desthio)	HPLC-MS/MS	KCP 5.2/13 Thies, S., 2015, Report No. M-536990-01-1, New study

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

Table 5.3-12: Validated methods for water (if appropriate) - Azoxystrobin

Component of residue definition: Azoxystrobin				
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 (Azoxystrobin)	GC-MSD	Robinson, N.J., 2000 / EU agreed Report No. RAM358/01
	Confirmatory		Two ions were used as qualifier. Confirmatory techniques are not necessary	
	Primary	0.1 µg/L (Azoxystrobin, Z-isomer R230310, metabolites R234886, R401553, R402173)	LC-MS/MS	KCP 5.2/14 Amic S., 2011, Report No. S11-02191, New study
	Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	
	ILV	0.1 µg/L (Azoxystrobin, Z-isomer R230310)	LC-MS/MS	KCP 5.2/15 Siekmann D., 2017, Report No. S17-01575, New study
Surface water (river and sea)	Primary	0.1 µg/L (Azoxystrobin)	GC-MSD	Robinson, N.J., 2000 / EU agreed Report No. RAM358/01
	Confirmatory		Two ions were used as qualifier. Confirmatory techniques are not necessary	
	Primary	0.1 µg/L (Azoxystrobin, Z-isomer R230310, metabolites R234886, R401553, R402173)	LC-MS/MS	KCP 5.2/14 Amic S., 2011, Report No. S11-02191, New study
	Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	
Ground water	Primary	0.1 µg/L (Azoxystrobin)	GC-MSD	Robinson, N.J., 2000 / EU agreed Report No.
	Confirmatory		Two ions were used as qualifier. Confirmatory techniques are not	

Component of residue definition: Azoxystrobin				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
			necessary.	RAM358/01
	Primary	0.1 µg/L (Azoxystrobin, Z-isomer R230310, metabolites R234886, R401553, R402173)	LC-MS/MS	KCP 5.2/4214 Amic S., 2011, Report No. S11-02191, New study
	Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	

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

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

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

Table 5.3-13: Validated methods for air (if appropriate) - Prothioconazole

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 Report No.: 00724
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.

Table 5.3-14: Validated methods for air (if appropriate) - Azoxystrobin

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.003 mg/m ³ (Azoxystrobin)	GC-MSD	Crawford, N., 2001 / EU agreed Report No. TMJ4658B
Confirmatory		Two ions were used as qualifier. Confirmatory techniques are not necessary	
Primary	2.2 µg/filter: equivalent to 0.003 µg/m ³ air aspired (Azoxystrobin)	LC-MS/MS	KCP 5.2/4416 Amic S., 2011, Report No. S11-02192, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	

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

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

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole and azoxystrobin 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. Nevertheless, an analytical method for the determination of prothioconazole-desthio in body fluids is presented below.

Table 5.3-15: Methods for body fluids and tissues (if appropriate) - Prothioconazole

Component of residue definition: NA			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	50 µg/L (Prothioconazole-desthio) in blood	HPLC-MS/MS	KCP 5.2/4517 Hoeppner, S., 2015, Report No. M-535874-02-1, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	

zRMS comments:

For a body fluids method for prothioconazole-desthio the limit of quantification was established at 0.05 mg/L, expressed as prothioconazole-desthio, but according to the SANTE/2020/12830, Rev.2, 14. February 2023, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues. It is necessary to supply the method for determining the residues of prothioconazole in body fluids with lower LOQ=0.01 mg/L at the renewal of the active substance and/or re-evaluation of plant production product (data gap). Please refer to the zRMS-PL conclusions presented in point 5.1.

Table 5.3-15: Methods for body fluids and tissues (if appropriate) - Azoxystrobin

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.05 µg/mL (Azoxystrobin and R234886 metabolite) in human plasma	HPLC-UV	Hall, M.G., 1999 / EU agreed
Confirmatory		LC-MS	
Primary	0.05 mg/L (Azoxystrobin and R230310 isomer) in human urine	LC-MS/MS	KCP 5.2/4618 Siekmann D., 2017, Report No. S17-01576, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	
Primary	0.05 µg/mL (Azoxystrobin and R234886 metabolite) in human plasma and urine	LC-MS/MS	KCP 5.2/4719 Amic S., 2011, Report No. S11-02193, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	
Primary	0.01 mg/L azoxystrobin in body fluids	LC-MS/MS	KCP 5.2/4820 Harper, H., 2022, Report No.8485925, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	
Primary	0.01 mg/kg (Azoxystrobin and R230310) in Bovine muscle meat	LC-MS/MS	KCP 5.2/07 Siekmann D., 2017, Report No. S17-01577, New study
Confirmatory		Two mass transitions were used. Confirmatory techniques are not necessary.	

5.3.2.8 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	Previously evaluated
KCP 5.1.1/01 (Submitted with confidential JK-CP)	Wang Q.	2021	Validation of Analytical Methodology for the Assay of Active Ingredient and Impurities in Prothioconazole 150 g/L + Azoxystrobin 150 g/L SC, CA3642, Report No. ABC-2021-018 Achiever Biochem Co., Ltd. GLP Unpublished	N	Nufarm	N
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 Agroscience Services Chem GmbH GLP Unpublished	N	Nufarm	Yes, in RR, Part B5 for CA3301/ Joust (01.2023)
KCP 5.1.2/01a	Heinz N.	2024	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole (PTZ) and its Metabolite PTZ-desthio in Different Matrices of Plant Origin Report No. S23-106298 Eurofins Agroscience Services EAG Laboratories GmbH GLP Unpublished	N	Nufarm	N
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 Agroscience Services Chem GmbH GLP Unpublished	N	Nufarm	Yes, in RR, Part B5 for CA3301/ Joust (01.2023)
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 Agroscience Services Chem GmbH GLP Unpublished	N	Nufarm	Yes, in RR, Part B5 for CA3301/ Joust (01.2023)
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	N	TDMG	Yes, in RR, Part B5 for

			Method 01062/M004, Report No. P 2383G, M-420638-01-1 PTRL Europe GmbH GLP Unpublished			CA3301/ Joust (01.2023)
KCP 5.1.2/05	North L.	2021	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole 250EC in Oilseed rape (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe 2019, Report No. S19-01269 Eurofins Agrosiences Services Ltd. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/06	North L.	2021	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole 250EC in Oilseed rape (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe 2020, Report No. S20-01046 Eurofins Agrosiences Services Ltd. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/07	North L.	2020	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole in Wheat (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe 2019, Report No. S19-01268 Eurofins Agrosiences Services Ltd. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/08	Freitag T.	2006	Analytical Method 00979 for the determination of residues of JAU 6476 3 hydroxy desthio, JAU 6476 4 hydroxy desthio, JAU 6476 5 hydroxy desthio, and JAU 6476 6 hydroxy desthio in/on Matrices of Plant Origin by HPLC-MS/MS, Report No. M-267072-01-1 Bayer CropScience AG GLP Unpublished	N	BAY	N
KCP 5.1.2/09	Freitag T.; Daniels M.	2009	Analytical Method 00979/M001 for the determination of residues of JAU 6476-a-hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio, and JAU 6476-6-hydroxy-desthio in/on matrices of plant origin by HPLC-MS/MS, Report No. M-328686-01-1 Bayer CropScience AG GLP Unpublished	N	BAY	N
KCP 5.1.2/10	Glaubitz J.; Hennes M.	2016	Modification M002 of the analytical method 00979/M001 for the determination of the metabolites JAU 6476-alpha-hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio and JAU 6476-6- hydroxy-desthio in plant matrices by HPLC-MS/MS, Report No. M-513336-02-1 Bayer CropScience AG GLP	N	BAY	N

			Unpublished			
KCP 5.1.2/11	Brumhard B.; Stuke S.	2016	Analytical method 01013 for the simultaneous determination of residues of the active Items BYF00587, prothioconazole, tebuconazole, trifloxystrobin and the metabolites BYF00587-desmethyl, JAU6476-desthio (SXX0665) and CGA321113 in/on plant material by HPLC-MS/MS, Report No. M-283439-04-1 Bayer CropScience AG GLP Unpublished	N	BAY	N
KCP 5.1.2/12	Bocksch S.	2023	A Semi-Field Study to Evaluate Potential Effects on the Honey Bee (Apis mellifera L.) After Two Applications of CA3301 and CA3642 in Winter Oil Seed Rape in Germany 2022, Report No. S21-00461 Eurofins Agrosience Services Ecotox GmbH GLP Unpublished	N	Nufarm	N
KCP 5.1.2/13	Gimeno I.	2022	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Honey Bee (Apis mellifera L.) chronic oral toxicity test (10-Day feeding) under laboratory conditions, Report No. S21-04081 Eurofins Agrosiences Services EcoChem GmbH. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/14	Gimeno I.	2022	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Honey Bee (Apis mellifera L.) larval toxicity test following repeated exposure under laboratory conditions, Report No. S21-04082 Eurofins Agrosiences Services EcoChem GmbH. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/15	Gimeno I.	2022	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Acute oral and contact Toxicity to the Bumblebee Bombus terrestris L., under laboratory conditions, Report No. S21-04083 Eurofins Agrosiences Services EcoChem GmbH. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/16	Huerta F.	2022	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Effects on the Seedling Emergence and Growth of Ten Non-Target Terrestrial Plant Species under Greenhouse Conditions, Report No. S21-04084 Eurofins Agrosiences Services EcoChem GmbH. GLP Unpublished	N	Nufarm	N
KCP 5.1.2/17	████	2023	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Acute toxicity to rainbow trout (Oncorhynchus mykiss), in a static 96-hour test, Report No. 20210195 ████ GLP Unpublished	Y	Nufarm	N
KCP 5.1.2/18	Dupont A.	2023	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Acute toxicity to Daphnia magna in a	N	Nufarm	N

			48-Hour Immobilization Test, Report No. 20210196 Innovative Environmental Services (IES) Ltd GLP Unpublished			
KCP 5.1.2/19	Dupont A.	202-3	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Effect on Skeletonema sp. In a 72-Hour Algal Growth-Inhibition Test, Report No. 20210197 Innovative Environmental Services (IES) Ltd GLP Unpublished	N	Nufarm	N
KCP 5.1.2/20	Bocksch S.	2008	Azoxystrobin (ICI5504) and Cyproconazole (SAN619)- Residues in honey following exposure of bees to treated winter oil-seed rape in Germany during 2007, Report No. T011298-06-REG Syngenta GLP Unpublished	N	Syngenta	Y EU level
KCP 5.1.2/21	Lebrun F.	2019	Magnitude of the residue of azoxystrobin in oilseed rape pollen and nectar Raw Agricultural Commodity after two foliar applications of ALB 121 in Southern Europe – 2018, Report No. 349-2018 Testapi GLP Unpublished	N	Albaugh Europe SARL	N
KCP 5.1.2/22	Appeltauer A.	2022	Azoxystrobin - Determination of Residues of Azoxystrobin and R230310 (z-isomer) in Honey after Two Applications of A12705B to Winter Oilseedrape at 5 Sites in Northern and Southern Europe in 2021, Report No. S11-01128 Eurofins Agrosience Services Ecotox GmbH GLP Unpublished	N	Syngenta Ltd.	N
KCP 5.1.2/23	Calvert A.	2024	CA3642 – Effectiveness of Cleaning Report No. 23/1610 Nufarm UK Limited GLP Unpublished	N	Nufarm	N
KCP 5.2/01	Kawa-Miszcak L.	2011	Validation of residue analytical method and storage stability of residue during storage of samples, Report No. PBBZ-2011/07/DPL Food Safety Laboratory GLP Unpublished	N	Nufarm	N
KCP 5.2/01b	Kawa-Miszcak L.	2011	Supplement A to final report Validation of residue analytical method and storage stability of residue during storage of samples, Report No. PBBZ-2011/07/DPL Food Safety Laboratory	N	Nufarm	N

			GLP Unpublished			
KCP 5.2/02	Lefresne S.	2011	Azoxystrobin and its metabolite Z-isomer – Independant laboratory validation (ILV) of an analytical method for the determination of residues in winter wheat (whole plant and grain) and oilseed rape (grain), Report No. NUFARM/AZO/11.01 GIRPA GLP Unpublished	N	Nufarm	N
KCP 5.2/03	Schulte, G.; Oel, D.	2014	Analytical method 01009 for the determination of residues of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4- dihydroxy-desthio, and JAU 6476-4,5-dihydroxy-desthio in/on matrices of animal origin by HPLC-MS/MS Report No. M-279725-03-1 Bayer CropScience AG, Monheim, Germany GLP Unpubilshed	N	Bayer Crop Science	Y in RR, Part B5 for GF 3307 (01.2023)
KCP 5.2/04	Bacher R.	2006	Independent laboratory validation of Bayer CropScience method No. 01009 for the determination of residues of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxy-desthio, and JAU 6476-4,5- 4,5-dihydroxy-desthio in/on Matrices of Animal Origin by HPLC-MS/MS Report No. M-279818-01-1 PTRL Europe GmbH, Ulm, Germany GLP Unpublished	N	Bayer Crop Science	Y in RR, Part B5 for GF 3307 (01.2023)
KCP 5.2/05	Kalathoor R.	2021	Development and validation of analytical methods for the determination of prothioconazole in different matrices , Report No. S20-09747 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	Y in RR, Part B5 for CA3301/Joust 250 EC (01.2023)
KCP 5.2/05b	Kalathoor R.	2021	Report Amendment 1 to Final report Development and validation of analytical methods for the determination of prothioconazole in different matrices , Report No. S20-09747 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	Y in RR, Part B5 for CA3301/Joust 250 EC (01.2023)
KCP 5.2/06	Greiner M.	2021	Independant laboratory validation of analytical methods for the determination of prothioconazole in honey, Report No. S21-02654 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	Y in RR, Part B5 for CA3301/Joust 250 EC (01.2023)

KCP 5.2/07	Siekman D.	2017	Laboratory validation of a method for the determination of Azoxystrobin and R230310 in Different matrices of animal origin, Report No. S17-01577 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	N
KCP 5.2/08	Meyer M.	2017	Independant laboratory validation of methods for the determination of azoxystrobin and R230310 in different matrices of animal origin , Report No. S17-02332 Eurofins Agroscience Services Chem SAS GLP Unpublished	N	Nufarm	N
KCP 5.2/09	Harper, H	2022	Azoxystrobin – Azoxystrobin (ICI5504) - Validation of the Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin and its Metabolite R230310 in Honey Matrices by LC-MS/MS, Report No. 8485926 GLP Unpublished	N	Syngenta	N
KCP 5.2/10	Homazaya, N.	2022	Azoxystrobin – Azoxystrobin - ILV of the Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin and its Metabolite R230310 in Honey Matrices by LC-MS/MS, Report No. 20210438 GLP Unpublished	N	Syngenta	N
KCP 5.2/11	Amic S.	2011	Validation of an analytical method for azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in soil, Report No. S11-02190 Eurofins, ADME BIOANALYSES GLP Unpublished	N	Nufarm	N
KCP 5.2/12	Krebber R.; Sandau C.	2015	Modification M002 of analytical method 01387 for the determination of various pesticides in drinking and surface water by HPLC-MS/MS, Report No. M-526061-01-1 Bayer CropScience AG, Monheim, Germany GLP Unpublished	N	Bayer Crop Science	Y in RR, Part B5 for ADM.03500.F.2.B (11.2022)
KCP 5.2/13	Thies S.	2015	Independent laboratory validation of the BCS analytical method 01387/M002 for the determination of various pesticides in surface water by HPLC-MS/MS, Report No. M-536990-01-1 Currenta GmbH & Co. OHG, Leverkusen, Germany GLP Unpublished	N	Bayer Crop Science	Y in RR, Part B5 for ADM.03500.F.2.B (11.2022)
KCP 5.2/14	Amic S.	2011	Validation of an analytical method for azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in water, Report No. S11-02191 Eurofins, ADME BIOANALYSES GLP	N	Nufarm	N

			Unpublished			
KCP 5.2/15	Siekman D.	2017	Independant validation of a method for the determination of azoxystrobin and R230310 in water, Report No. S17-01575 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	N
KCP 5.2/16	Amic S.	2011	Validation of an analytical method for azoxystrobin in air, Report No. S11-02192 Eurofins, ADME BIOANALYSES GLP Unpublished	N	Nufarm	N
KCP 5.2/17	Hoepfner S.	2015	Validation of the BCS analytical method 01471 for the determination of prothiconazole-desthio in body fluid by HPLC-MS/MS Report No. M-535874-02-1 Currenta GmbH & Co. OHG, Leverkusen, Germany GLP Unpublished	N	Bayer Crop Science	Y in RR, Part B5 for GF 3307 (01.2023)
KCP 5.2/18	Siekman D.	2017	Laboratory validation of a method for the determination of azoxystrobin and R230310 in body fluids, Report No. S17-01576 Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Nufarm	N
KCP 5.2/19	Amic S.	2011	Validation of an analytical method for azoxystrobin and its metabolite R234886 in human plasma and urine, Report No. S11-02193 Eurofins, ADME BIOANALYSES GLP Unpublished	N	Nufarm	N
KCP 5.2/20	Harper, H.	2022	Azoxystrobin – Azoxystrobin (ICI5504) - Validation of Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin in Body Fluid by LC-MS/MS, Report No 8485925 GLP Unpublished	N	Syngenta	N

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	Ryan J.,	1996	ICIA5504 and R230310: Validation of a method for the determination of residues in animal tissue, eggs and milk,	N	Syngenta

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
	Sapiets A.		Report No. RJ1809B Syngenta GLP Unpublished		
CP 5.1.2 CP 5.2	Johnson R.I.	2000	Residue analytical method for the analysis of azoxystrobin, R230310, R234886, R401553 and R402173 [in soil] Report No. 269/03 Jealott's Hill research Station, UK Not GLP Unpublished	N	Syngenta
CP 5.1.2 CP 5.2	Robinson N.J.	2000	Analytical method for the determination of residues of azoxystrobin in water Report No. RAM 358/01 Jealott's Hill research Station, UK Not GLP Unpublished	N	BAY
CP 5.1.2 CP 5.2	Crawford N.	2001	Azoxystrobin: Validation of an analytical method for the determination of residues in air Report No. TMJ4658B Jealott's Hill research Station, UK Not GLP Unpublished	N	BAY
CP 5.1.2	Hall M.G.	1999	Azoxystrobin and R234886: Determination in Human and Animal plasma by LC-UV and LC-MS, Report No. CTL/R/1401 GLP Unpublished	N	Syngenta
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-dethio in materials of plant and animal origin Report No. 00086/M033 Dr. Specht & Partner GLP	N	BAY
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

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 5.2	Lister N.	1999	Azoxystrobin: Validation of RAM 305/01 for the determination of Azoxystrobin and R230310 in crops Report No. RJ2770B Jealott's Hill Research station, UK GLP Unpublished	N	Syngenta
CP 5.2	Kang J.	2003	Independent laboratory validation of method RAM 305/02 for the determination of residues of Azoxystrobin and R230310 in leafy crops, brassicae and roots/tuber crops Report No. CEMR-1708 v3 CEMAS Ltd. GLP Unpublished	N	Syngenta
CP 5.2	Chaggar S.	2004	Azoxystrobin (ICI5504) and R230310: Validation of residue analytical method RAM 305/03 for the determination of residues in crops Report No. RJ3552B Jealott's Hill Research station, UK GLP Unpublished	N	Syngenta
CP 5.2	Croucher A.	2002	Independent laboratory validation of SOP RAM 305/02 Analytical method for the determination of residues in crops (brassicae, maize and roots crops) Report No. 1983/029-D2419 Covance Laboratories Ltd GLP Unpublished	N	Syngenta
CP 5.2	Heinemann, O.	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 Bayer AG GLP Unpublished	N	BAY
CP 5.2	Heinemann, O.	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 Bayer AG GLP Unpublished	N	BAY

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 5.2	Dubey L.	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 Battelle Geneva Research Centres GLP Unpublished	N	BAY
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 5.2	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 5.2	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 5.2	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

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for prothioconazole and azoxystrobin

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

A 2.1.1.1 Description of analytical methods for the determination of residues in plant matrices

A 2.1.1.1.1 Prothioconazole and its metabolites in different matrices of plant origin

A 2.1.1.1.1.1 Method validation 1

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust (January 2023). This method has not been reassessed in the framework of this application.</p> <p>The conclusions of the assessment are presented below:</p> <p><i>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.</i></p> <p><i>Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes and matrices at each level.</i></p> <p><i>The study is acceptable.</i></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: Phenomenex Luna C18(2) 100Å, 150 mm x 2 mm, 5 µm)
Mobile phase A: Methanol
Mobile phase B: Water + 10 mM ammonium acetate

Time (min)	% A	% B
0	50	50
4	95	5
6	95	5
6.1	50	50
8	50	50

Flow: 0.6 L/min

Column temperature: 50°C

Injection volume: 20 µL

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 (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
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		

Table A 2: Recovery results from method validation of prothioconazole-desthio using the analytical method

Prothioconazole-desthio							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)

Transition m/z 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 m/z 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 3: Characteristics for the analytical method used for validation of prothioconazole residues in plant origin matrices

	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 A4 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 4: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Prothioconazole	Wheat (grain)	342 → 100 m/z (Quantification)	$y = 269019.6651 x + 7609.2371$, $r = 0.9998$
		342 → 125 m/z (Confirmation)	$y = 162668.0296 x + 7599.8426$, $r = 0.9994$
	Grapes	342 → 100 m/z (Quantification)	$y = 84468.6526 x - 331.1023$, $r = 0.9994$

	Oilseed rape (seeds)	342 → 125 m/z (Confirmation)	$y = 54979.3614 x + 1061.5103, r = 0.9995$
		342 → 100 m/z (Quantification)	$y = 154482.6713 x - 2423.1974, r = 0.9995$
		342 → 125 m/z (Confirmation)	$y = 96810.3583 x - 1013.4184, r = 0.9999$
	Bean (dry)	342 → 100 m/z (Quantification)	$y = 103352.8037 x - 470.5023, r = 0.9998$
		342 → 125 m/z (Confirmation)	$y = 64989.1355 x - 191.1264, r = 0.9999$
	Cucumber	342 → 100 m/z (Quantification)	$y = 250876.3629 x + 2976.5787, r = 0.9997$
		342 → 125 m/z (Confirmation)	$y = 155001.3629 x + 1419.2871, r = 0.9993$
Prothioconazole-desthio	Wheat (grain)	312 → 70 m/z (Quantification)	$y = 379803.3489 x - 3175.7042, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y = 199091.9003 x + 706.4512, r = 0.9999$
	Grapes	312 → 70 m/z (Quantification)	$y = 1007320.8723 x + 76813.3437, r = 0.9977$
		312 → 125 m/z (Confirmation)	$y = 489156.5421 x + 23235.4945, r = 0.9976$
	Oilseed rape (seeds)	312 → 70 m/z (Quantification)	$y = 137251.9470 x + 1668.9220, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y = 69533.4891 x + 2055.4582, r = 0.9997$
	Bean (dry)	312 → 70 m/z (Quantification)	$y = 246299.4548 x - 3555.2148, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y = 128550.2336 x - 906.3960, r = 0.9997$
	Cucumber	312 → 70 m/z (Quantification)	$y = 384992.9907 x + 8337.7142, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y = 202167.4455 x + 2048.1244, r = 0.9998$

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. Confirmatory method (if required) Confirmatory data presented in initial method validation.

A 2.1.1.1.2 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.3 Independent Laboratory Validation

Comments of zRMS:	The analytical method S16-04434 was successfully independently validated for the determination of residues of prothioconazole (PTZ) and its metabolite PTZ-desthio in different plant matrices (high water content, high acid, high oil content and high protein/high
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	starch content) with LOQ of 0.01 mg/kg. Mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met. The method is acceptable.
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Reference:	KCP 5.1.2/01a
Report	Independent Laboratory Validation of an Analytical Method for the Determination of Prothioconazole (PTZ) and its Metabolite PTZ-desthio in Different Matrices of Plant Origin, Heinz. N., 2024, report number S23-106298
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability:	Yes

The purpose of this study was to independently validate the analytical method in study S16-04434 (KCP 5.1.2/01) for the determination of relevant residues of prothioconazole and prothioconazole-desthio in/on matrices of plant origin by HPLC-MS/MS.

Materials and methods

Methods were applied as in KCP 5.1.2/01, Report No. S16-04434. A minor modification to the original method was performed in which the stock solution was prepared in acetonitrile instead of acetone. The stability of the analytes prothioconazole and prothioconazole-desthio in acetonitrile under refrigerated conditions was successfully proven. Consequently, this modification has no influence on the final results.

Results and discussions

Table A 2: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 342→100 (Proposed for Quantification)							
Wheat (Grain)	0.01	85, 88, 88, 87, 88	87	2	5	89	4
	0.1	89, 91, 87, 96, 94	91	4	5		
Beans (Dry)	0.01	78, 75, 80, 83, 89	81	6	5	83	7
	0.1	91, 88, 83, 75, 87	85	7	5		
Oilseed Rape (Seeds)	0.01	92, 86, 93, 84, 76	86	8	5	86	6
	0.1	88, 87, 87, 85, 82	86	3	5		
Grapes	0.01	87, 88, 87, 90, 90	89	2	5	91	4
	0.1	92, 90, 96, 95, 98	94	3	5		
Cucumber	0.01	87, 89, 85, 81, 88	86	4	5	94	9
	0.1	102, 103, 102, 95, 104	101	3	5		
Transition <i>m/z</i> 342→125 (Proposed for Confirmation)							
Wheat (Grain)	0.01	88, 91, 88, 89, 92	89	2	5	91	3
	0.1	90, 92, 89, 97, 95	93	4	5		
Beans (Dry)	0.01	81, 73, 83, 81, 88	81	7	5	84	7
	0.1	92, 88, 85, 76, 89	86	7	5		
Oilseed Rape (Seeds)	0.01	91, 87, 90, 85, 82	87	4	5	87	4
	0.1	88, 91, 85, 86, 85	87	3	5		
Grapes	0.01	92, 98, 94, 97, 96	95	2	5	96	4
	0.1	96, 89, 99, 97, 101	96	5	5		
Cucumber	0.01	87, 86, 88, 86, 95	88	4	5	94	8

	0.1	95, 104, 104, 92, 106	100	6	5		
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Table A 2: Recovery results from method validation of prothioconazole-desthio using the analytical method

Prothioconazole-desthio							
Matrix	Fortificati on Level	Recovery	Mean Recovery	Rel. Std. Dev.	Replicate s	Overall Mean Recovery	Overall Rel. Std. Dev.
	(mg/kg)	(%)	(%)	(%)		(%)	(%)
Transition m/z 312→70 (Proposed for Quantification)							
Wheat (Grain)	0.01	90, 92, 88, 88, 90	90	2	5	95	6
	0.1	100, 100, 95, 102, 101	100	3	5		
Beans (Dry)	0.01	97, 88, 94, 92, 97	94	4	5	96	4
	0.1	99, 98, 98, 95, 98	98	2	5		
Oilseed Rape (Seeds)	0.01	75, 74, 75, 71, 71	73	3	5	74	3
	0.1	77, 76, 75, 76, 75	76	1	5		
Grapes	0.01	91, 94, 89, 97, 99	94	5	5	96	4
	0.1	96, 94, 99, 99, 100	98	3	5		
Cucumber	0.01	105, 104, 104, 104, 104	104	0	5	107	3
	0.1	108, 111, 106, 110, 112	109	2	5		
Transition m/z 312→125 (Proposed for Confirmation)							
Wheat (Grain)	0.01	89, 93, 88, 89, 93	90	3	5	94	5
	0.1	99, 100, 93, 101, 100	98	3	5		
Beans (Dry)	0.01	95, 89, 93, 92, 99	94	4	5	95	3
	0.1	98, 97, 98, 95, 97	97	1	5		
Oilseed Rape (Seeds)	0.01	75, 71, 75, 73, 75	74	3	5	75	2
	0.1	76, 75, 75, 77, 74	75	1	5		
Grapes	0.01	92, 94, 91, 99, 98	95	4	5	96	4
	0.1	95, 93, 100, 99, 101	98	4	5		
Cucumber	0.01	102, 105, 102, 104, 101	103	2	5	106	3
	0.1	108, 110, 106, 108, 112	109	2	5		

Table A 3: Characteristics for the analytical method used for validation of prothioconazole residues in plant origin matrices

	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 (performed with 1/x weighting). The correlation coefficients, r obtained were > 0.99. Please see table A4 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. Prothioconazole (PTZ): Insignificant (< 20 %) for the matrices wheat (grain), beans (dry) and grapes. Significant (≥ 20 %) for the matrices oilseed rape (seeds) and cucumber. Prothioconazole-desthio (PTZ-desthio): Significant (≥ 20 %) for all investigated matrices wheat (grain), beans (dry), grapes, oilseed rape (seeds) and cucumber.
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.

	Prothioconazole
Stability of standards and extracts	Stock solution in Acetonitrile: stable 68 days between 1-10°C for prothioconazole and stable 50 days between 1-10°C for prothioconazole-desthio. Calibration solutions in methanol/water (2/3, v/v) for prothioconazole and prothioconazole-desthio (0.15-10 ng/mL): stable for 8 days between 1-10°C. Extract stable for 7 days between 1-9°C (except for prothioconazole in bean – analysis directly after extraction recommended).

Table A 4: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Prothioconazole	Wheat (grain)	342 → 100 m/z (Quantification)	$y = 3.91E+05 * x + -7.67E+003, r = 0.9997$
		342 → 125 m/z (Confirmation)	$y_i = 2.49E+05 * x + -4.56E+03, r = 0.9997$
	Bean (dry)	342 → 100 m/z (Quantification)	$y_i = 3.77E+05 * x + 1.63E+004, r = 0.9999$
		342 → 125 m/z (Confirmation)	$y_i = 2.40E+05 * x + 1.04E+04, r = 0.9998$
	Grapes	342 → 100 m/z (Quantification)	$y_i = 4.07E+05 * x + 1.25E+004, r = 0.9993$
		342 → 125 m/z (Confirmation)	$y_i = 2.54E+05 * x + 3.38E+03, r = 0.9997$
	Cucumber	342 → 100 m/z (Quantification)	$y_i = 2.75E+05 * x + -3.98E+03, r = 1.0000$
		342 → 125 m/z (Confirmation)	$y_i = 1.78E+05 * x + -6.47E+03, r = 0.9996$
	Oilseed rape (seeds)	342 → 100 m/z (Quantification)	$y_i = 1.14E+05 * x + 7.4E+003, r = 0.9989$
		342 → 125 m/z (Confirmation)	$y_i = 7.34E+04 * x + 5.41E+03, r = 0.9987$
Prothioconazole-desthio	Wheat (grain)	312 → 70 m/z (Quantification)	$y_i = 2.42E+05 * x + 1.07E+004, r = 0.9999$
		312 → 125 m/z (Confirmation)	$y_i = 1.47E+05 * x + 6.56E+03, r = 0.9999$
	Bean (dry)	312 → 70 m/z (Quantification)	$y_i = 2.32E+05 * x + 3.14E+003, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y_i = 1.4E+05 * x + 1.83E+03, r = 0.9999$
	Grapes	312 → 70 m/z (Quantification)	$y_i = 2.71E+05 * x + 5.66E+003, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y_i = 1.63E+05 * x + 3.75E+03, r = 0.9999$
	Cucumber	312 → 70 m/z (Quantification)	$y_i = 2.57E+05 * x + 6.39E+003, r = 0.9997$
		312 → 125 m/z (Confirmation)	$y_i = 1.53E+05 * x + 3.28E+03, r = 0.9997$
	Oilseed rape (seeds)	312 → 70 m/z (Quantification)	$y_i = 1.12E+05 * x + 3.36E+003, r = 0.9998$
		312 → 125 m/z (Confirmation)	$y_i = 6.69E+04 * x + 1.21E+03, r = 0.9999$

Conclusion

An independent laboratory validation of this analytical method has been performed on plant matrices (high water content, high acid, high oil content and high protein/high starch content). The method has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.2 requirements were fulfilled for an independent laboratory validation. 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 2.1.1.1.1.4

Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.5 Method validation 2

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust (January 2023). This method has not been reassessed in the framework of this application.</p> <p>The conclusions of the assessment are presented below:</p> <p><i>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-α-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.</i></p> <p><i>All mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes and matrices at each level.</i></p> <p><i>The study is acceptable.</i></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: Phenomenex Kinetex PFP 100A, 100
mm x 3 mm, 2.6 µm
Mobile phase A: Acetonitrile
Mobile phase B: Water + 0.2% v/v acetic acid
Flow: 0.7 L/min
Column temperature: 50°C
Injection volume: 25 µL

Time (min)	% A	% B
0	20	80
6	30	70
8	90	10
9	90	10
9.1	20	80
11	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 (<i>m/z</i>)
Prothioconazole- α -hydroxy-desthio	328 \rightarrow 70 [#]
	328 \rightarrow 141
Prothioconazole-3-hydroxy-desthio	328 \rightarrow 70 [#]
	328 \rightarrow 141
Prothioconazole-4-hydroxy-desthio	328 \rightarrow 70 [#]
	328 \rightarrow 141
Prothioconazole-5-hydroxy-desthio	328 \rightarrow 70 [#]
	328 \rightarrow 141
Prothioconazole-6-hydroxy-desthio	328 \rightarrow 70 [#]
	328 \rightarrow 141

#: used for quantification but both transitions are interchangeable.

Results and discussions

Table A 5: Recovery results from method validation of prothioconazole metabolites using the analytical method

Prothioconazole- α -hydroxy-desthio							
Analyte: Prothioconazole- α -hydroxy-desthio		Final determination as: Prothioconazole- α -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	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 m/z 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	0.01	129, 99, 112, 84, 108	106	16	5	106	12

rape (seeds)	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 m/z 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 m/z 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	0.01	99, 91, 100, 75, 90	91	11	5	93	10
	0.1	88, 105, 89, 104, 86	94	9.8	5		

(seeds)							
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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 <i>m/z</i> 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		

Prothioconazole-desthio-3-glucoside		
Analyte:	Final determination as:	Residues calculated as:

Prothioconazole- desthio-3-glucoside		Prothioconazole- desthio-3-glucoside		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		
<div> <div>Prothioconazole-desthio-3-glucoside</div> <div> <div>Analyte:</div> <div>Prothioconazole- desthio-3-glucoside</div> </div> <div> <div>Final determination as:</div> <div>Prothioconazole- desthio-3-glucoside</div> </div> <div> <div>Residues calculated as:</div> <div>Prothioconazole-desthio</div> </div> </div>							
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-dethio-4-glucoside							
Analyte: Prothioconazole- dethio-4-glucoside		Final determination as: Prothioconazole- dethio-4-glucoside			Residues calculated as: Prothioconazole-dethio		
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	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 m/z 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		

Analyte:	Prothioconazole-desthio-6-glucoside	
	Final determination as:	Residues calculated as:

Prothioconazole- desthio-6-glucoside		Prothioconazole- desthio-6-glucoside		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	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 m/z 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 6: 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, 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 A7 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 - α -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- α -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 - α -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 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 7: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Prothioconazole- α -hydroxy-desethio	Wheat (whole plant)	328→70 m/z (Quantification)	$y = 34319.0296 x + 4179.6946, r = 0.9988$
		328→141 m/z (Confirmation)	$y = 11376.1941 x + 1492.0611, r = 0.9988$
	Wheat (grain)	328→70 m/z (Quantification)	$y = 35976.0930 x + 2192.4459, r = 0.9990$
		328→141 m/z (Confirmation)	$y = 11805.7114 x + 916.8273, r = 0.9989$
	Wheat (straw)	328→70 m/z (Quantification)	$y = 14862.5221 x + 391.9694, r = 0.9995$
		328→141 m/z (Confirmation)	$y = 4798.1804 x + 458.3559, r = 0.9986$
	Oilseed rape (seeds)	328→70 m/z (Quantification)	$y = 15828.8097 x + 2350.3173, r = 0.9997$
		328→141 m/z (Confirmation)	$y = 5148.3952 x + 817.8952, r = 0.9996$
Prothioconazole-3-hydroxy-desethio	Wheat (whole plant)	328→70 m/z (Quantification)	$y = 32515.6432 x + 6074.0157, r = 0.9968$
		328→141 m/z (Confirmation)	$y = 12912.6106 x + 2319.8469, r = 0.9978$
	Wheat (grain)	328→70 m/z (Quantification)	$y = 39927.3187 x + 4616.7082, r = 0.9983$
		328→141 m/z (Confirmation)	$y = 15333.6871 x + 1641.0340, r = 0.9974$
	Wheat (straw)	328→70 m/z (Quantification)	$y = 14741.4708 x + 448.5433, r = 0.9998$
		328→141 m/z (Confirmation)	$y = 5473.5911 x + 428.3996, r = 0.9991$
	Oilseed rape (seeds)	328→70 m/z (Quantification)	$y = 14084.0030 x + 2883.0456, r = 0.9994$
		328→141 m/z (Confirmation)	$y = 5331.0083 x + 946.9468, r = 0.9993$
Prothioconazole-4-hydroxy-desethio	Wheat (whole plant)	328→70 m/z (Quantification)	$y = 43145.8175 x + 10394.8518, r = 0.9945$
		328→141 m/z (Confirmation)	$y = 30558.1501 x + 7170.7571, r = 0.9955$
	Wheat (grain)	328→70 m/z (Quantification)	$y = 54104.1193 x + 8317.8887, r = 0.9954$
		328→141 m/z (Confirmation)	$y = 37628.3043 x + 5606.5273, r = 0.9959$
	Wheat (straw)	328→70 m/z (Quantification)	$y = 18411.0184 x + 1480.9083, r = 0.9993$
		328→141 m/z (Confirmation)	$y = 12737.6801 x + 910.1181, r = 0.9993$
	Oilseed rape (seeds)	328→70 m/z (Quantification)	$y = 12878.8982 x + 1661.0520, r = 0.9993$
		328→141 m/z (Confirmation)	$y = 8913.7731 x + 1108.2783, r = 0.9994$
Prothioconazole-5-hydroxy-desethio	Wheat (whole plant)	328→70 m/z (Quantification)	$y = 35861.0564 x + 6127.5497, r = 0.9978$
		328→141 m/z (Confirmation)	$y = 14577.8115 x + 2680.1892, r = 0.9980$
	Wheat (grain)	328→70 m/z (Quantification)	$y = 46383.7756 x + 5591.7687, r = 0.9966$
		328→141 m/z (Confirmation)	$y = 18779.3278 x + 2134.4164, r = 0.9974$
	Wheat (straw)	328→70 m/z (Quantification)	$y = 16392.2669 x + 1165.1554, r = 0.9997$
		328→141 m/z (Confirmation)	$y = 6674.6020 x + 278.8368, r = 0.9988$

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

Analyte	Matrix	Transition	Linearity data
		(Confirmation)	
	Oilseed rape (seeds)	328→70 m/z (Quantification)	$y = 3990.5181 x + 58.3848, r = 0.9994$
		328→141 m/z (Confirmation)	$y = 7189.8913 x - 52.9434, r = 0.9996$

Conclusion

This analytical method for the determination of prothioconazole- α -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- α -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 2.1.1.1.6 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.7 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 Triazole derivative metabolites in Agricultural commodity

A 2.1.1.1.2.1 Method validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust (January 2023). This method has not been reassessed in the framework of this application.</p> <p>The conclusions of the assessment are presented below:</p> <p><i>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.</i></p> <p><i>All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20 % for all analytes and matrices at each level.</i></p> <p><i>The study is acceptable.</i></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: Phenomenex SecurityGuard™ for C18 HPLC
Column: Thermo Hypercarb, 100 mm x 3 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

Time (min)	% A	% B
0	0	100
3	0	100
6.5	80	20
6.51	0	100
9	0	100

Analytical conditions for confirmation

System: Series 1290 HPLC, Agilent Technologies
Pre-column: Phenomenex SecurityGuard™ for C18 HPLC

Column: Synergi 4u Polar-RP 80A, 150 mm x 4.6 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

Time (min)	% A	% B
0	30	70
4	80	20
4.01	30	70
8	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 8: 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 m/z 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 m/z (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	0.01	114, 93, 115, 115, 91	106	12	5	100	13

rape	0.1	93, 82, 82, 110, 108	95	14	5		
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Triazole lactic acid							
Matrix	Fortification Level	Recovery	Mean Recovery	Rel. Std. Dev.	Replicates	Overall Mean Recovery	Overall Rel. Std. Dev.
	(mg/kg)	(%)	(%)	(%)		(%)	(%)
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	0.01	101, 112, 118, 100, 106	107	7.1	5	107	6.1

rape	0.1	111, 97, 109, 104, 112	107	5.8	5		
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Table A 9: 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 10 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 10: Linearity of detector response

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

Analyte	Matrix	Transition	Linearity data
		157→88 m/z (Confirmation) 162→75 m/z (ISTD)	$y = 0.0920 x + 0.0044, r = 0.9974$
		157→70 m/z (Quantification) 162→75 m/z (ISTD)	$y = 0.2028 x + 0.0135, r = 0.9992$
	Grapes	157→88 m/z (Confirmation) 162→75 m/z (ISTD)	$y = 0.0932 x + 0.0050, r = 0.9995$
		157→70 m/z (Quantification) 162→75 m/z (ISTD)	$y = 0.1793 x + 0.0270, r = 0.9914$
	Oilseed rape	157→88 m/z (Confirmation) 162→75 m/z (ISTD)	$y = 0.0808 x + 0.0121, r = 0.9885$
		157→70 m/z (Quantification) 162→75 m/z (ISTD)	

Analyte	Matrix	Transition (m/z)	Linearity data
TLA	Wheat (grain)	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1208 x - 0.0001, r = 0.9961$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1085 x + 0.0050, r = 0.9995$
	Wheat (straw)	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9968$
	Barley (grain)	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1215 x + 0.0146, r = 0.9977$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1558 x - 0.0035, r = 0.9993$
	Barley (straw)	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1199 x + 0.0001, r = 0.9995$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1160 x + 0.0015, r = 0.9997$
	Grapes	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9988$
	Oilseed rape	158→70 m/z (Quantification) 163→75 m/z (ISTD)	$y = 0.1158 x + 0.0024, r = 0.9975$
		158→70 m/z (Quantification) 163→75 m/z (ISTD)	Confirmatory method $y = 0.1145 x + 0.0007, r = 0.9988$
TAA	Wheat (grain)	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1325 x + 0.0039, r = 0.9961$
		128→70 m/z (Quantification) 133→75 m/z (ISTD)	Confirmatory method $y = 0.1230 x + 0.0097, r = 0.9989$
	Wheat (straw)	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1243 x + 0.00668, r = 0.9978$
		128→70 m/z (Quantification) 133→75 m/z (ISTD)	Confirmatory method $y = 0.1262 x + 0.0032, r = 0.9992$

Analyte	Matrix	Transition (m/z)	Linearity data
	Barley (grain)	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1205 x + 0.0077$, $r = 0.9993$
		128→70 m/z (Quantification)133→75 m/z (ISTD)	Confirmatory method $y = 0.1297 x - 0.0015$, $r = 0.9998$
	Barley (straw)	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1342 x - 0.0033$, $r = 0.9996$
		128→70 m/z (Quantification) 133→75 m/z (ISTD)	Confirmatory method $y = 0.1311 x + 0.0034$, $r = 0.9996$
	Grapes	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1243 x + 0.0068$, $r = 0.9978$
		128→70 m/z (Quantification) 133→75 m/z (ISTD)	Confirmatory method $y = 0.1262 x + 0.0032$, $r = 0.9992$
	Oilseed rape	128→70 m/z (Quantification) 133→75 m/z (ISTD)	$y = 0.1229 x + 0.0079$, $r = 0.9979$
		128→70 m/z (Quantification) 133→75 m/z (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 2.1.1.1.2.2 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. 4 23 February 2022, 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 2.1.1.1.3 Triazole derivative metabolites in plant materials

A 2.1.1.1.3.1 Method validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust (January 2023). This method has not been reassessed in the framework of this application.</p> <p>The conclusions of the assessment are presented below: <i>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.</i></p> <p><i>Mean recoveries were within the 70 - 110% range for most matrix (16) and analyte (4) combinations. Few average recoveries were above 110% but < 120%, obviously caused by analyte present in control samples requiring background correction and supported by acceptable relative standard deviations (RSDs) and thus considered acceptable.</i></p> <p><i>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 < 30%, caused by endogenous TA present in the untreated sample requiring background subtraction. Nevertheless, these results are considered acceptable.</i></p> <p><i>The method meets in general all guideline criteria and is acceptable.</i></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 x 3 mm., 3 µm, 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

Time (min)	% A	% B
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0	95	5
1	95	5
1.30	60	40
3	60	40
3.01	5	95
5	5	95
5.51	95	5
8.50	95	5

Analytical conditions for confirmation

System: Series 1200HPLC, Agilent Technologies
Column: Thermo Hypercarb column, 100 mm x 4.6 mm.,
5 µm, 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

Time (min)	% A	% B
0	95	5
3	95	5
5	60	40
8	60	40
8.01	5	95
11	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 11: Recovery results from method validation of triazole derivative metabolites using the analytical method

1,2,4-triazole						
Mass Transition 70→43 m/z (Proposed for Quantification)						
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						
Mass Transition 157→70 m/z (Proposed for Quantification)						
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

Triazole alanine						
Mass Transition 157→70 m/z (Proposed for Quantification)						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
	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						
Mass Transition 128→70 m/z (Proposed for Quantification)						
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

Triazole acetic acid						
Mass Transition 128→70 m/z (Proposed for Quantification)						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
	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						
Mass Transition 158→70 m/z (Proposed for Quantification)						
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		
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

Triazole lactic acid						
Mass Transition 158→70 m/z (Proposed for Quantification)						
Matrix	Fortification level [mg/kg]	No of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	RSD [%]
	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 32: 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 triazolylalanine (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 triazolylalanine (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 > 0.99.</p> <p>Representative equations of the calibration line:</p> <p>1,2,4-triazole (C18 column): $y = 0.837 x + 0.00158$ ($r = 0.9993$) ($n = 8$)</p> <p>Triazolylalanine (C18 column): $y = 1.95 x - 0.000528$ ($r = 0.9987$) ($n = 8$)</p> <p>Triazole acetic acid (C18 column): $y = 1.01 x + 0.0018$ ($r = 0.9991$) ($n = 8$)</p> <p>Triazole Lactic acid (C18 column): $y = 1.07 x - 0.00333$ ($r = 0.9997$) ($n = 8$)</p> <p>Triazolylalanine (Hypercarb column): $y = 1.61 x + 0.00488$ ($r = 0.9999$) ($n = 8$)</p> <p>Triazole Lactic acid (Hypercarb column): $y = 0.801 x + 0.0073$ ($r = 0.9972$) ($n = 8$)</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 2.1.1.1.3.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.3.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.4 Prothioconazole-desthio, prothioconazole metabolites and triazole metabolites in oilseed rape

A 2.1.1.1.4.1 Method validation 1

Comments of zRMS:	<p>Specimens extraction and determination of residues of PTZ-desthio were performed according to the multiresidue QuEChERS method that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber in S16-04434 with the LOQ of 0.01 mg/kg.</p> <p>Specimens extraction and determination of residues of PTZ-α-hydroxy-desthio, PTZ-3-, -4-, -5- and -6-hydroxy-desthio were performed according to the analytical method described in S16-04435 that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (whole plant, grain and straw), and oilseed rape (seed) with the LOQ of 0.01 mg/kg.</p> <p>Specimens extraction and determination of residues of TDMs were performed according to the analytical method described in S15-03542 that was previously validated according to the SANCO/825/00, rev. 8.1 for wheat and barley (grain and straw), grape (bunches) and oilseed rape with the LOQ of 0.01 mg/kg.</p> <p>In this study the reduced method validation for the matrices oilseed rape was performed. The LOQ was 0.01 mg/kg for all analytes and for all matrices.</p> <p>All mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes and matrices at each level.</p> <p>The methods are acceptable.</p>
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Reference:	KCP 5.1.2/05
Report	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole 250EC in Oilseed rape (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe 2019, North L., 2021, Report No. S19-01269
Guideline(s):	SANCO/3029/99 rev.4, SANCO/825/00 rev 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

For prothioconazole-desthio determination, samples were extracted with acetonitrile after adding water using multi-residue QuEChERS method. Quantification was performed by use of LC-MS/MS detection. The limit of quantification was 0.01 mg/kg.

For prothioconazole- α -hydroxy-desthio, prothioconazole-3-, 4-, 5-, 6-hydroxy-desthio determination, samples were extracted with acetonitrile/water (4/1 v/v) mixture and hydrolysed before purification on a SPE cartridge. Quantification was performed by use of LC-MS/MS detection. The limit of quantification

was 0.01 mg/kg (calculated as prothioconazole-desthio).

For triazole derivate metabolites (TDMs) determination, samples were extracted with methanol/water (4/1 v/v) mixture. After internal standard addition, the extract is evaporated to the aqueous remainder. Quantification was performed by use of LC-MS/MS detection. The limit of quantification was 0.01 mg/kg.

Analytical conditions for prothioconazole-desthio

Chromatographic conditions

System: 1260 Infinity Binary LC system, Agilent technologies

Column: Luna C18(2) 100A, 150 mm x 2 mm, 5 µm

Mobile phase A: Methanol

Mobile phase B: Water containing 10 mM ammonium acetate

Flow: 0.6 mL/min

Column temperature: 50°C

Injection volume: 20 µL

Time (min)	% A	% B
0	50	50
4.0	95	5
6.0	95	5
6.1	50	50
8.0	50	50

Divert valve: 0.0min to 1.0min to waste, 1.0 min to 6.5min to MS, 6.5 min to 8.0 min to waste

Retention time: Prothioconazole-desthio: About 4.2 min

Mass spectrometric conditions

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

Ionisation type: Electrospray ionisation (ESI, Turbolon Spray)

Polarity: Positive/Negative ion switching mode

Capillary voltage: 5500V (pos) – 4500V (neg)

Ionspray turbo heater: 500°C

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

Mass transitions (prothioconazole-desthio): 312 → 70 pos (m/z) for quantification, 312 → 125 pos (m/z) for confirmation

Analytical conditions for prothioconazole metabolites

Chromatographic conditions

System: 1200 Binary Rapid Resolution LC system, Agilent technologies

Column: Kinetex PFP 100A, 100 mm x 3 mm, 2.6 µm

Mobile phase A: Acetonitrile

Mobile phase B: Water + 0.2% v/v acetic acid

Flow: 0.7 mL/min

Column temperature: 50°C

Injection volume: 25 µL

Time (min)	% A	% B
0	20	80
6.0	30	70
8.0	90	10
9.0	90	10
9.10	20	80
11.0	20	80

Divert valve: 0.0min to 3.0min to waste, 3.0 min to 8.5min to MS, 8.5 min to 11.0 min to waste

Retention time:

Prothioconazole- α -hydroxy-desthio: About 4.5 min

Prothioconazole-3-hydroxy-desthio: About 6.2 min

Prothioconazole-4-hydroxy-desthio: About 7.0 min
Prothioconazole-5-hydroxy-desthio: About 7.3 min
Prothioconazole-6-hydroxy-desthio: About 8.0 min

Mass spectrometric conditions

System: API 4000 system, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)

Polarity: Positive ion mode

Ion spray voltage: 5500 V

Ionspray turbo heater: 600°C

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

Mass transition

Prothioconazole- α -hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Prothioconazole-3-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Prothioconazole-4-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Prothioconazole-5-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Prothioconazole-6-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Analytical conditions for triazole metabolites

Chromatographic conditions (quantification)

System: Series 1290 HPLC system, Agilent technologies

Pre-column: Phenomenex SecurityGuard for C18 HPLC

Column: Thermo Hypercarb, 100 mm x 3 mm, 5.0 μ m

Mobile phase A: Methanol + 0.5% v/v formic acid

Mobile phase B: Water + 0.5% v/v formic acid

Flow: 0.6 mL/min

Column temperature: 60°C

Injection volume: 20 μ L

Time (min)	% A	% B
0	0	100
3.0	0	100
6.5	80	20
6.51	0	100
9.0	0	100

Divert valve: 0.0min to 0.7min to waste, 0.7 min to 6.5min to MS

Retention time:

1,2,4-triazole: About 1.4 min

Triazole alanine: About 1.8 min

Triazole acetic acid: About 4.8 min

Triazole lactic acid: About 5.0 min

Chromatographic conditions (confirmation)

System: Series 1290 HPLC system, Agilent technologies

Pre-column: Phenomenex SecurityGuard for C18 HPLC

Column: Synergi 4 μ Polar-RP 80A, 150 mm x 4.6 mm, 4.0 μ m

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

Time (min)	% A	% B
0	30	70
4.0	80	20
4.01	30	70
8.00	30	70

Divert valve: 0.0min to 1.5min to waste, 1.5 min to 6.9min to MS

Retention time:

1,2,4-triazole: About 2.7 min

Triazole acetic acid: About 2.7 min

Triazole lactic acid: About 2.6 min

Mass spectrometric conditions

System: SCIEX TripleQuad 6500 system, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)

Polarity: Positive ion mode

Ion spray voltage: 4000 V

Ionspray turbo heater: 350°C

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

Mass transition

1,2,4-triazole: 70 → 43 (m/z) for quantification

1,2,4-triazole (ISTD): 75 → 46 (m/z)

Triazole alanine: 157 → 70 (m/z) for quantification, 157 → 88 (m/z) for confirmation

Triazole alanine (ISTD): 162 → 75 (m/z)

Triazole acetic acid: 128 → 70 (m/z)

Triazole acetic acid (ISTD): 133 → 75 (m/z)

Triazole lactic acid: 158 → 70 (m/z)

Triazole lactic acid (ISTD): 163 → 75 (m/z)

Results and discussions

Table A 43: Recovery results from method validation of prothioconazole-desthio using the analytical method

Prothioconazole-desthio							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 312 → 70 <i>m/z</i> (Proposed for Quantification)							
Oilseed rape (whole plant)	0.01	86, 87, 103, 97, 96, 85	92	8.0	6	91	7.4
	0.1	96, 87, 99, 85, 85	90	7.3	5		
Oilseed rape (plant)	0.01	86, 90, 90	89	2.6	3	90	2.0
	0.1	90, 91, 90	90	0.6	3		

Recoveries are without any blank correction

Analyte: PTZ-desthio, Final determination: PTZ-desthio, Residues calculated as: PTZ-desthio

Table A 54: Recovery results from method validation of prothioconazole metabolites using the analytical method

Prothioconazole-α-hydroxy-desthio							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 328 → 70 <i>m/z</i> (Proposed for Quantification)							
Oilseed rape (whole plant)	0.01	110, 104, 110, 110, 109, 110, 110, 110	109	1.9	8	109	1.4
	0.1	110, 110, 110, 109, 108,	110	0.7	8		

Analyte: PTZ- α -OH-desthio, Final determination: PTZ- α -OH-desthio, Residues calculated as: PTZ-desthio

Analyte: PTZ-6-OH-desthio. Final determination: PTZ-6-OH-desthio. Residues calculated as: PTZ-desthio

[illegible]

Oilseed rape (whole plant)	0.01	105, 108, 105, 115, 93	105	7.6	5	105	5.6
	0.1	101, 105, 109, 110, 103	106	3.6	5		
Oilseed rape (plant)	0.01	83, 103, 88	91	11	3	95	8.5
	0.1	97, 103, 94	98	4.7	3		
Triazole lactic acid [Transition <i>m/z</i> 158 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	93, 101, 92, 119, 108	103	11	5	100	10
	0.1	84, 95, 94, 103, 108	97	9.5	5		
Oilseed rape (plant)	0.01	94, 85, 70	83	15	3	91	13
	0.1	91, 97, 106	98	7.7	3		

Recoveries are corrected for the mean peak area of the control sample extracts

Analyte: 1,2,4-triazole, Final determination: 1,2,4-triazole, Residues calculated as: 1,2,4-triazole

Analyte: Triazole alanine, Final determination: Triazole alanine, Residues calculated as: Triazole alanine

Analyte: Triazole acetic acid, Final determination: Triazole acetic acid, Residues calculated as: Triazole acetic acid

Analyte: Triazole lactic acid, Final determination: Triazole lactic acid, Residues calculated as: Triazole lactic acid

Table A 16: Characteristics for the analytical method used for validation of prothioconazol-desthio, prothioconazole metabolites and triazole derivate metabolites in oilseed rape

	Prothioconazole-desthio	Prothioconazole metabolites	Triazole derivate metabolites
Specificity	<p>The analyte PTZ desthio was determined by use LC-MS/MS. One mass transition was evaluated. A second mass transition was monitored for confirmation peak identity.</p> <p>At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention time of PTZ-desthio of the control sample materials that were used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ. Blank correction was not necessary.</p>	<p>The analytes of PTZ metabolites were determined by use LC-MS/MS. For each analyte, one mass transition was evaluated. A second mass transition was monitored for confirmation peak identity.</p> <p>At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention times of PTZ metabolites analytes of the control sample materials that were used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ. Blank correction was not necessary.</p>	<p>The analytes of Triazole metabolites were determined by use LC-MS/MS. For each analyte one mass transition was evaluated. For each of the internal standards of 1,2,4-Triazole, TA, TAA and TLA one mass transition was evaluated.</p> <p>At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention time of 1,2,4-Triazole of the control sample materials did not exceed 30 % of the LOQ. The blank values at the expected retention times of TA, TAA and TLA of the control sample materials did exceed 30 % of the LOQ. The situation was considered unavoidable. No other appropriate source of control samples of wheat could be found. Correction for blank values was performed even if they were below 30 % of the LOQ.</p>
Calibration (type, number of data points)	<p>The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A17 below.</p>	<p>The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were</p>	<p>The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y =$</p>

	Prothioconazole-desthio	Prothioconazole metabolites	Triazole derivate metabolites
		> 0.99. Please see table A17 below.	a*x + b). The correlation coefficients, r obtained were > 0.99. Please see table A17 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL (n = 7), This range corresponds to a fortification level of 0.003 mg/kg to 0.20 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL (n = 7), This range corresponds to a fortification level of 0.003 mg/kg to 0.20 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.	Linearity was confirmed over the calibration range 0.3 – 400.0 ng/mL (n = 10), This range corresponds to a fortification level of 0.003 mg/kg to 4.0 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. For 1,2,4-Triazole, TA, TAA and TLA calibration standards contained the internal standards at a constant concentration level of 8.0 ng/mL.
Assessment of matrix effects is presented	No	No	No
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg. The LOD is 0.003 mg/kg.	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg for all analytes. The LOD is 0.003 mg/kg.	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg for all analytes. The LOD is 0.003 mg/kg.
Stability of standards and extracts	The stability of the analyte PTZ-desthio in the final extracts of oilseed rape (seed) upon storage at typically 1 °C to 10 °C for eight days was demonstrated in S16-04434 study. Furthermore for a high water matrix (cucumber) a storage stability for nine days was demonstrated.	The stability of the analytes in the final extracts of oilseed rape (seed) upon storage at typically 1 °C to 10 °C for 14 days was demonstrated in S16-04435 study. Furthermore for a high water matrix (wheat (whole plant)) a storage stability for 20 days was demonstrated.	Due to the use of internal standards the stability of the TDMs in the final extracts was not assessed in S15-03542 study.

Table A 17: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Prothioconazole-desthio	Oilseed rape (whole plant)	312 → 70 m/z (Quantification)	y = 647490.5231 x + 16339.6513, r = 0.9879 (n = 7)
	Oilseed rape (seeds)	312 → 70 m/z (Quantification)	y = 472526.6618 x + 2252.0669, r = 0.9999 (n = 7)
Prothioconazole-α-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	y = 41996.9169 x + 2770.3094, r = 0.9993 (n = 7)
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	y = 2760.5661 x – 67.8695, r = 0.9985 (n = 7)

Prothioconazole-3-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 48308.0617 x + 3848.5938, r = 0.9983 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3096.2952 x + 2.5334, r = 0.9995 (n = 7)$
Prothioconazole-4-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 54000.5054 x + 4840.9329, r = 0.9987 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3280.1567 x - 31.0251, r = 0.9999 (n = 7)$
Prothioconazole-5-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 46362.6485 x + 3355.0597, r = 0.9990 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3156.6187 x - 31.8410, r = 0.9993 (n = 7)$
Prothioconazole-6-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 52974.7789 x + 2708.8776, r = 0.9989 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3608.6985 x - 143.8308, r = 0.9997 (n = 7)$
1,2,4-triazole	Oilseed rape (whole plant)	70 → 43 m/z (Quantification)	$y = 0.1053 x + 0.0030, r = 0.9985 (n = 10)$
	Oilseed rape (seeds)	70 → 43 m/z (Quantification)	$y = 0.1232 x - 0.0006, r = 0.9989 (n = 10)$
Triazole alanine	Oilseed rape (whole plant)	157 → 70 m/z (Quantification)	$y = 0.1520 x - 0.0001, r = 0.9994 (n = 10)$
	Oilseed rape (seeds)	157 → 70 m/z (Quantification)	$y = 0.1449 x + 0.0039, r = 0.9999 (n = 10)$
Triazole acetic acid	Oilseed rape (whole plant)	128 → 70 m/z (Quantification)	$y = 0.1306 x + 0.0001, r = 0.9998 (n = 10)$
	Oilseed rape (seeds)	128 → 70 m/z (Quantification)	$y = 0.1420 x + 0.0126, r = 0.9998 (n = 10)$
Triazole lactic acid	Oilseed rape (whole plant)	158 → 70 m/z (Quantification)	$y = 0.2802 x - 0.0177, r = 0.9977 (n = 10)$
	Oilseed rape (seeds)	158 → 70 m/z (Quantification)	$y = 0.2429 x + 0.0196, r = 0.9999 (n = 10)$

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1 for the determination of prothioconazole-desthio, prothioconazole- α -hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio, as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in oilseed rape (whole plant and seed). In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for all analytes in all matrices.

A 2.1.1.1.4.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.4.2 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.4.3 Method validation 2

Comments of zRMS:	Specimens extraction and determination of residues of PTZ-desthio were performed
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	<p>according to the multiresidue QuEChERS method that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber in S16-04434 with the LOQ of 0.01 mg/kg.</p> <p>Specimens extraction and determination of residues of PTZ-α-hydroxy-desthio, PTZ-3-, -4-, -5- and -6-hydroxy-desthio were performed according to the analytical method described in S16-04435 that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (whole plant, grain and straw), and oilseed rape (seed) with the LOQ of 0.01 mg/kg.</p> <p>Specimens extraction and determination of residues of TDMs were performed according to the analytical method described in S15-03542 that was previously validated according to the SANCO/825/00, rev. 8.1 for wheat and barley (grain and straw), grape (bunches) and oilseed rape with the LOQ of 0.01 mg/kg.</p> <p>In this study the reduced method validation for the matrices oilseed rape was performed. The LOQ was 0.01 mg/kg for all analytes and for all matrices.</p> <p>The accuracy and precision of the method during sample analysis were considered to be acceptable since single recoveries were in the range of 60 - 120% and the mean recoveries at each fortification level were in the range of 70 – 110% with relative standard deviation(s) below 20 % for all combinations of matrices and analytes.</p> <p>The methods are acceptable.</p>
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Reference:	KCP 5.1.2/06
Report	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole 250EC in Oilseed rape (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe, North L., 2021, Report N°S20-01046
Guideline(s):	SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

For prothioconazole-desthio determination, samples were extracted with acetonitrile after adding water using multi-residue QuEChERS method. Quantification was performed by use of LC-MS/MS detection. The limit of quantification was 0.01 mg/kg.

For prothioconazole- α -hydroxy-desthio, prothioconazole-3-, 4-, 5-, 6-hydroxy-desthio determination, samples were extracted with acetonitrile/water (4/1 v/v) mixture and hydrolysed before purification on a SPE cartridge. Quantification was performed by use of LC-MS/MS detection. The limit of quantification was 0.01 mg/kg (calculated as prothioconazole-desthio).

For triazole derivate metabolites (TDMs) determination, samples were extracted with methanol/water (4/1 v/v) mixture. After internal standard addition, the extract is evaporated to the aqueous remainder. Quantification was performed by use of LC-MS/MS detection. The limit of quantification was 0.01 mg/kg.

Analytical conditions for prothioconazole-desthio

Chromatographic conditions

System: 1290 Infinity II High speed pump LC system, Agilent technologies

Column: Luna C18(2) 100A, 150 mm x 2 mm, 5 μ m

Mobile phase A: Methanol

Mobile phase B: Water containing 10 mM ammonium acetate

Flow: 0.6 mL/min

Column temperature: 50°C

Injection volume: 3 μ L

	% A	% B
0 min	50	50
4.0 min	95	5
6.0 min	95	5
6.1 min	50	50
8.0 min	50	50

Divert valve: 0.0min to 1.0min to waste, 1.0 min to 6.5min to MS, 6.5 min to 8.0 min to waste

Retention time: Prothioconazole-desthio: About 3.1 min

Mass spectrometric conditions

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

Ionisation type: Electrospray ionisation (ESI, Turbolon Spray)

Polarity: Positive/Negative ion switching mode

Capillary voltage: 5500V (pos) – 4500V (neg)

Ionspray turbo heater: 500°C

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

Mass transitions

Prothioconazole-desthio: 312 → 70 pos (m/z) for quantification, 312 → 125 pos (m/z) for confirmation

Prothioconazole: 342 → 100 pos (m/z) for quantification, 342 → 125 pos (m/z) for confirmation

Analytical conditions for prothioconazole metabolites

Chromatographic conditions

System: 1200 Binary Rapid Resolution LC system, Agilent technologies

Column: Kinetex PFP 100A, 100 mm x 3 mm, 2.6 µm

Mobile phase A: Acetonitrile

Mobile phase B: Water + 0.2% v/v acetic acid

Flow: 0.7 mL/min

Column temperature: 50°C

Injection volume: 25 µL

Time (min)	% A	% B
0	20	80
6.0	30	70
8.0	90	10
9.0	90	10
9.10	20	80
11.0	20	80

Divert valve: 0.0min to 3.0min to waste, 3.0 min to 8.5min to MS, 8.5 min to 11.0 min to waste

Retention time:

Prothioconazole- α -hydroxy-desthio: About 4.5 min

Prothioconazole-3-hydroxy-desthio: About 6.2 min

Prothioconazole-4-hydroxy-desthio: About 7.0 min

Prothioconazole-5-hydroxy-desthio: About 7.3 min

Prothioconazole-6-hydroxy-desthio: About 8.0 min

Mass spectrometric conditions

System: API 4000 system, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)

Polarity: Positive ion mode

Ion spray voltage: 5500 V

Ionspray turbo heater: 600°C

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

Mass transition

Prothioconazole- α -hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation
Prothioconazole-3-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation
Prothioconazole-4-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation
Prothioconazole-5-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation
Prothioconazole-6-hydroxy-desthio: 328 \rightarrow 70 (m/z) for quantification, 328 \rightarrow 141 (m/z) for confirmation

Analytical conditions for triazole metabolites

Chromatographic conditions (quantification)

System: Series 1290 HPLC system, Agilent technologies
Pre-column: Phenomenex SecurityGuard for C18 HPLC
Column: Thermo Hypercarb, 100 mm x 3 mm, 5.0 μ m
Mobile phase A: Methanol + 0.5% formic acid
Mobile phase B: Water + 0.5 % (v/v) formic acid
Flow: 0.6 mL/min
Column temperature: 60°C
Injection volume: 20 μ L

Time (min)	% A	% B
0	0	100
3.0	0	100
6.5	80	20
6.51	0	100
9.0	0	100

Divert valve: 0.0min to 0.7min to waste, 0.7 min to 6.5min to MS, 6.5min to 9.0min to waste
Retention time:

1,2,4-triazole: About 1.6 min
Triazole alanine: About 2.2 min
Triazole acetic acid: About 5.5 min
Triazole lactic acid: About 5.5min

Chromatographic conditions (confirmation)

System: Series 1290 HPLC system, Agilent technologies
Pre-column: Phenomenex SecurityGuard for C18 HPLC
Column: Synergi 4 μ Polar-RP 80A, 150 mm x 4.6 mm, 4.0 μ m
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 μ L

Time (min)	% A	% B
0	30	70
4.0	80	20
4.01	30	70
8.00	30	70

Divert valve: 0.0min to 1.5min to waste, 1.5 min to 4.0min to MS, 4.0min to 8.0min to waste
Retention time:

1,2,4-triazole: About 2.7 min
Triazole acetic acid: About 2.7 min
Triazole lactic acid: About 2.6 min

Mass spectrometric conditions

System: SCIEX TripleQuad 6500 system, SCIEX (Triple quadrupole mass spectrometer)

Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)

Polarity: Positive ion mode

Ion spray voltage: 4000 V

Ionspray turbo heater: 350°C

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

Mass transition

1,2,4-triazole: 70 → 43 (m/z) for quantification

1,2,4-triazole (ISTD): 75 → 46 (m/z)

Triazole alanine: 157 → 70 (m/z) for quantification

Triazole alanine (ISTD): 162 → 75 (m/z)

Triazole acetic acid: 128 → 70 (m/z)

Triazole acetic acid (ISTD): 133 → 75 (m/z)

Triazole lactic acid: 158 → 70 (m/z)

Triazole lactic acid (ISTD): 163 → 75 (m/z)

Results and discussions

Table A 78: Recovery results from method validation of prothioconazole, prothioconazole-desthio and prthioconazole metabolites using the analytical method

Prothioconazole							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 342 → 100 m/z (Proposed for Quantification)							
Oilseed rape (whole plant)	0.01	81, 84, 70, 72, 76	77	7.7	5	75	6.2
	0.1	76, 70, 72, 74, 78	74	4.3	5		
Oilseed rape (seeds)	0.01	71, 70, 74	72	2.9	3	74	4.3
	0.1	75, 77, 78	77	2.0	3		

Prothioconazole-desthio							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 312 → 70 m/z (Proposed for Quantification)							
Oilseed rape (whole plant)	0.01	95, 98, 109, 102, 101	101	5.2	5	101	4.1
	0.1	102, 96, 104, 104, 99	101	3.4	5		
Oilseed rape (seeds)	0.01	89, 85, 101	92	9.1	3	89	8.6
	0.1	93, 81, 82	85	7.8	3		

Recoveries are without any blank correction

Analyte: Prothioconazole, Final determination: Prothioconazole, Residues calculated as: PTZ-desthio

Analyte: PTZ-desthio, Final determination: PTZ-desthio, Residues calculated as: PTZ-desthio

Prothioconazole-α-hydroxy-desthio							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 328 → 70 m/z (Proposed for Quantification)							
Oilseed rape (whole plant)	0.01	82, 95, 95, 81, 88, 76, 85, 92, 86, 87	87	7.1	10	86	7.9
	0.1	78, 94, 93, 79, 90, 75, 81, 84, 94, 78	85	8.8	10		
Oilseed rape (seeds)	0.01	95, 99, 108	101	6.6	3	98	5.2
	0.1	94, 96, 97	96	1.6	3		

Recoveries are without any blank correction

Analyte: PTZ-α-OH-desthio, Final determination: PTZ-α-OH-desthio, Residues calculated as: PTZ-desthio

Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Prothioconazole-3-hydroxy-desthio [Transition <i>m/z</i> 328 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	87, 85, 73, 82, 91, 80, 96, 86, 85, 83	85	7.3	10	85	6.3
	0.1	83, 94, 90, 81, 91, 81, 81, 84, 86, 82	85	5.6	10		
Oilseed rape (seeds)	0.01	108, 91, 92	97	9.8	3	94	7.7
	0.1	87, 93, 93	91	3.8	3		
Prothioconazole-4-hydroxy-desthio [Transition <i>m/z</i> 328 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	72, 94, 85, 78, 96, 75, 76, 85, 96, 85	84	11	10	85	8.7
	0.1	77, 92, 92, 81, 91, 83, 82, 81, 93, 82	85	6.9	10		
Oilseed rape (seeds)	0.01	91, 96, 99	95	4.2	3	94	4.5
	0.1	87, 95, 95	92	5.0	3		
Prothioconazole-5-hydroxy-desthio [Transition <i>m/z</i> 328 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	73, 71, 78, 70, 84, 86, 71, 74, 87, 82	78	8.6	10	81	8.7
	0.1	80, 89, 91, 79, 95, 85, 79, 82, 88, 80	85	6.7	10		
Oilseed rape (seeds)	0.01	87, 100, 102	96	8.5	3	93	7.9
	0.1	83, 93, 95	90	7.1	3		
Prothioconazole-6-hydroxy-desthio [Transition <i>m/z</i> 328 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	72, 95, 74, 73, 84, 77, 78, 78, 85, 80	80	8.7	10	79	9.0
	0.1	82, 85, 91, 70, 86, 68, 72, 76, 85, 76	79	9.8	10		
Oilseed rape (plant)	0.01	79, 97, 98	91	12	3	90	7.9
	0.1	90, 86, 88	88	2.3	2		

Recoveries are without any blank correction

Analyte: PTZ-3-OH-desthio, Final determination: PTZ-3-OH-desthio, Residues calculated as: PTZ-desthio

Analyte: PTZ-4-OH-desthio, Final determination: PTZ-4-OH-desthio, Residues calculated as: PTZ-desthio

Analyte: PTZ-5-OH-desthio, Final determination: PTZ-5-OH-desthio, Residues calculated as: PTZ-desthio

Analyte: PTZ-6-OH-desthio, Final determination: PTZ-6-OH-desthio, Residues calculated as: PTZ-desthio

Table A 89: Recovery results from method validation of triazole derivate metabolites using the analytical method

Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
1,2,4-triazole [Transition <i>m/z</i> 70 → 43 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	113, 104, 96, 92, 93, 90, 98	98	8.2	7	101	7.3
	0.1	104, 101, 96, 99, 107, 107, 113	104	5.5	7		
Oilseed rape (seeds)	0.01	101, 84, 64, 98, 88, 70	83	17	6	84	15
	0.1	99, 81, 67, 102, 85, 78	85	15	6		
Triazole alanine [Transition <i>m/z</i> 157 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	100, 89, 107, 105, 77, 89, 83	93	12	7	95	10
	0.1	99, 100, 102, 110, 86, 89, 96	97	8.3	7		
Oilseed rape (seeds)	0.01	110, 104, 100, 105, 94	103	5.8	5	97	14
	0.05	78, 113, 101, 94, 71	91	19	5		
Triazole acetic acid [Transition <i>m/z</i> 128 → 70 <i>m/z</i> (Quantification)]							
Oilseed rape (whole plant)	0.01	104, 98, 94, 96, 102, 100, 103	100	3.8	7	100	5.0
	0.1	98, 94, 97, 103, 106, 95, 111	101	6.3	7		
Oilseed rape (seeds)	0.01	107, 109, 111, 101, 99	105	4.9	5	106	4.5
	0.1	111, 105, 110, 111, 100	107	4.5	5		

Triazole lactic acid [Transition m/z 158 → 70 m/z (Quantification)]							
Oilseed rape (whole plant)	0.01	83, 91, 101, 95, 90, 91, 99	93	6.5	7	93	4.9
	0.1	93, 94, 88, 90, 97, 93, 93	93	3.1	7		
Oilseed rape (seeds)	0.01	111, 106, 96, 90, 108	102	8.7	5	102	6.8
	0.1	96, 101, 105, 110, 98	102	5.5	5		

Recoveries are corrected for the mean peak area of the control sample extracts

Analyte: 1,2,4-triazole, Final determination: 1,2,4-triazole, Residues calculated as: 1,2,4-triazole

Analyte: Triazole alanine, Final determination: Triazole alanine, Residues calculated as Triazole alanine

Analyte: Triazole acetic acid, Final determination: Triazole acetic acid, Residues calculated as: Triazole acetic acid

Analyte: Triazole lactic acid, Final determination: Triazole lactic acid, Residues calculated as: Triazole lactic acid

Table A 20: Characteristics for the analytical method used for validation of prothioconazole, prothioconazol-desthio, prothioconazole metabolites and triazole derivate metabolites in oilseed rape

	Prothioconazole Prothioconazole-desthio	Prothioconazole metabolites	Triazole derivate metabolites
Specificity	The analyte prothioconazole and PTZ desthio were determined by use LC-MS/MS. One mass transition was evaluated. A second mass transition was monitored for confirmation peak identity. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention time of prothioconazole and PTZ-desthio of the control sample materials that were used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ. Blank correction was not necessary.	The analytes of PTZ metabolites were determined by use LC-MS/MS. For each analyte, one mass transition was evaluated. A second mass transition was monitored for confirmation peak identity. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention times of PTZ metabolites analytes of the control sample materials that were used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ. Blank correction was not necessary.	The analytes of Triazole metabolites were determined by use LC-MS/MS after addition of internal standard. For each analytes one mass transition was evaluated. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention times of TA, TAA and TLA of the control sample materials did exceed 30 % of the LOQ. The situation was considered unavoidable. No other appropriate source of control samples of oilseed rape could be found. Correction for blank values was performed for all control matrices with residues below and above 30 % of the LOQ.
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A21 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A21 below.	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A21 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL (n = 7). This range corresponds to a fortification level of 0.003 mg/kg to 0.20 mg/kg and thus covers the range from no more	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL (n = 7). This range corresponds to a fortification level of 0.003 mg/kg to 0.20 mg/kg	Linearity was confirmed over the calibration range 0.3 – 400.0 ng/mL (n = 10). This range corresponds to a fortification level of

	Prothioconazole Prothioconazole-desthio	Prothioconazole metabolites	Triazole derivate metabolites
	than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.	and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.	0.003 mg/kg to 4.0 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. For 1,2,4-Triazole, TA, TAA and TLA calibration standards contained the internal standards at a constant concentration level of 8.0 ng/mL.
Assessment of matrix effects is presented	No	No	No
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg. The LOD is 0.003 mg/kg.	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg for all analytes. The LOD is 0.003 mg/kg.	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01mg/kg for all analytes. The LOD is 0.003 mg/kg.
Stability of standards and extracts	The stability of the analytes prothioconazole and PTZ-desthio in the final extracts of oilseed rape (seed) upon storage at typically 1 °C to 10 °C for eight days was demonstrated in S16-04434 study. Furthermore for a high water matrix (cucumber) a storage stability for nine days was demonstrated.	The stability of the analytes in the final extracts of oilseed rape (seed) upon storage at typically 1 °C to 10 °C for 23 days was demonstrated in S16-04435 study. Furthermore for a high water matrix (wheat (whole plant)) a storage stability for 20 days was demonstrated.	Due to the use of internal standards the stability of the TDMs in the final extracts was not assessed in S15-03542 study.

Table A 21: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Prothioconazole	Oilseed rape (whole plant)	342 → 100 m/z (Quantification)	$y = 44291.7362 x - 1727.8241, r = 0.9998 (n = 7)$
	Oilseed rape (seeds)	342 → 100 m/z (Quantification)	$y = 46258.2259 x + 1732.6113, r = 0.9999 (n = 7)$
Prothioconazole-desthio	Oilseed rape (whole plant)	312 → 70 m/z (Quantification)	$y = 68243.1135 x + 3060.1466, r = 0.9998 (n = 7)$
	Oilseed rape (seeds)	312 → 70 m/z (Quantification)	$y = 47284.0536 x + 1089.9960, r = 0.9999 (n = 7)$
Prothioconazole-α-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 1624.3771 x + 58.4788, r = 0.9991 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3467.1468 x - 141.7804, r = 0.9997 (n = 7)$
Prothioconazole-3-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 1719.9090 x + 43.4892, r = 0.9991 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3831.4531 x - 249.6037, r = 0.9991 (n = 7)$
Prothioconazole-4-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 1584.0283 x + 17.9494, r = 0.9998 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 4421.5416 x - 259.4404, r = 0.9997 (n = 7)$

Prothioconazole-5-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 1781.9459x - 50.1227, r = 0.9981 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 5048.6530x - 326.0861, r = 0.9995 (n = 7)$
Prothioconazole-6-hydroxy-desthio	Oilseed rape (whole plant)	328 → 70 m/z (Quantification)	$y = 2029.5072x + 10.2333, r = 0.9979 (n = 7)$
	Oilseed rape (seeds)	328 → 70 m/z (Quantification)	$y = 3293.9045x - 146.7935, r = 0.9994 (n = 7)$
1,2,4-triazole	Oilseed rape (whole plant)	70 → 43 m/z (Quantification)	$y = 0.0175x - 0.0030, r = 0.9998 (n = 10)$
	Oilseed rape (seeds)	70 → 43 m/z (Quantification)	$y = 0.0147x - 0.0009, r = 0.9995 (n = 10)$
Triazole alanine	Oilseed rape (whole plant)	157 → 70 m/z (Quantification)	$y = 0.0209x - 0.0018, r = 0.9997 (n = 10)$
	Oilseed rape (seeds)	157 → 70 m/z (Quantification)	$y = 0.0200x - 0.0005, r = 0.9996 (n = 10)$
Triazole acetic acid	Oilseed rape (whole plant)	128 → 70 m/z (Quantification)	$y = 0.0135x - 0.0007, r = 0.9999 (n = 10)$
	Oilseed rape (seeds)	128 → 70 m/z (Quantification)	$y = 0.0126x - 0.0002, r = 1.0000 (n = 10)$
Triazole lactic acid	Oilseed rape (whole plant)	158 → 70 m/z (Quantification)	$y = 0.0199x - 0.0003, r = 0.9997 (n = 10)$
	Oilseed rape (seeds)	158 → 70 m/z (Quantification)	$y = 0.0237x - 0.0006, r = 0.9999 (n = 10)$

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1 for the determination of prothioconazole-desthio, prothioconazole- α -hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio, as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in oilseed rape (whole plant and seed). In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for all analytes in all matrices.

A 2.1.1.1.4.4 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.4.5 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.5 Prothioconazole-desthio, prothioconazole metabolites and triazole metabolites in wheat

A 2.1.1.1.5.1 Method validation

Comments of zRMS:	Specimens extraction and determination of residues of PTZ-desthio were performed according to the multiresidue QuEChERS method that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (grain), grapes, oilseed rape (seed), bean (dry) and cucumber in S16-04434 with the LOQ of 0.01 mg/kg.
	Specimens extraction and determination of residues of PTZ- α -hydroxy-desthio, PTZ-3-, -4-

	<p>, -5- and -6-hydroxy-desthio were performed according to the analytical method described in S16-04435 that was previously validated according to the SANCO/3029/99 rev. 4 and SANCO/825/00, rev. 8.1 for wheat (whole plant, grain and straw), and oilseed rape (seed) with the LOQ of 0.01 mg/kg.</p> <p>Specimens extraction and determination of residues of TDMs were performed according to the analytical method described in S15-03542 that was previously validated according to the SANCO/825/00, rev. 8.1 for wheat and barley (grain and straw), grape (bunches) and oilseed rape with the LOQ of 0.01 mg/kg.</p> <p>The analytical methods were validated for the determination of all analytes in wheat (whole plant, grain, straw, ears and rest of plant) according to SANCO/3029/99, rev.4 during analysis. All mean recovery values (corrected for apparent blank residues, if necessary) at fortification levels of LOQ and 10x LOQ comply with the standard acceptance criteria of the guidance document with evaluation of one mass transition.</p> <p>The LOQ was 0.01 mg/kg for all analytes and for all matrices.</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 methods are acceptable.</p>
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Reference:	KCP 5.1.2/07
Report	Determination of residues of Prothioconazole-desthio (sum of isomers) after two applications of Prothioconazole in Wheat (outdoor) at 4 sites in Northern Europe and 4 sites in Southern Europe, 2020, North L. Report No. S19-01268
Guideline(s):	SANCO/3029/99 rev. 4, SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

In this study, a validation was conducted for prothioconazole-desthio (PTZ-desthio), PTZ- α -hydroxy-desthio, PTZ-3-, -4-, -5- and -6-hydroxy-desthio as well as the triazole derivative metabolites (TDMs) 1,2,4-Triazole, triazole alanine (TA), Triazole acetic acid (TAA) and Triazole lactic acid (TLA) in raw agricultural commodity wheat.

Prothioconazole-desthio

Sample of wheat were extracted with acetonitrile after addition of water. A salt mixture containing magnesium sulfate, sodium chloride, trisodium citrate dihydrate and disodium hydrogen citrate sesquihydrate was added and sample was shaken and centrifuged. Aliquot was then diluted with methanol/water (2/3, v/v). If necessary, final extracts were diluted with control matrix-extract to be within the calibration range.

Prothioconazole metabolites

Sample of wheat were extracted with acetonitrile/water (4/1, v/v). Celite was added and sample was stirred and filtered and Buchner funnel. Filtered residues were washed 3 times with acetonitrile/water (4/1, v/v) and sucked to dryness. The extract was evaporated to the aqueous remainder. The remainder was hydrolysed and cleaned-up with SPE technique. Extracts obtained were diluted in acetonitrile. If necessary, final extracts were diluted with control matrix-extract to be within the calibration range.

Triazole metabolites

Sample of wheat were extracted with methanol after addition of water and filtered. Internal standard was added. The solution was evaporate to the aqueous remainder. Water was added to adjust the volume and sonicated. The sample was filtered using 45 μ m cellulose single use

filter. If necessary, final extracts were diluted with matrix-extract to be within the calibration range

Quantification was performed by use LC-MS/MS using matrix matched calibration.

Analytical conditions for Prothioconazole-desthio

LC conditions

System: 1260 Infinity Binary LC system, Agilent Technologies

Column: Phenomenex Luna C18(2), 100A, 150 mm x 2.0 mm, 5µm

Column temperature: 50°C

Flow: 0.6 mL/min

Mobile phase

Eluent A: Methanol

Eluent B: Water containing 10mM ammonium acetate

Gradient:

Time (min)	% A	% B
0.0	50	50
4.0	95	5
6.0	95	5
6.1	50	50
8.0	50	50

Divert valve:

0.0 min to 1.0 min to waste

1.0 min to 6.5 min to MS

6.5 min to 8.0 min to waste

Injection volume: 20 µL

Retention time:

Prothioconazole-desthio: About 4.2 min

MS conditions

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

Ionisation type: Electrospray ionisation (ESI, Turbolon Spray)

Polarity: Positive/negative ion switching mode

Scan type: MS/MSn Multiple reaction monitoring (MRM)

Ionspray turbo heater: 500°C

Capillary voltage (IS) 5500 V (pos) – 4500 V (neg)

Prothioconazole-desthio: 312 → 70 m/z proposed for quantification and 312 → 125 m/z proposed for confirmation

Analytical conditions for Prothioconazole metabolites

LC conditions

System: 1200 Binary Rapid Resolution LC system, Agilent Technologies

Column: Phenomenex Kinetex PFP, 100A, 100 mm x 3.0 mm, 2.6µm

Column temperature: 50°C

Flow: 0.7 mL/min

Mobile phase

Eluent A: Acetonitrile

Eluent B: Water + 0.2% v/v acetic acid

Gradient:

Time (min)	% A	% B
0.00	20	80
6.00	30	70
8.00	90	10
9.00	90	10
9.10	20	80
11.00	20	80

Divert valve:

0.0 min to 3.0 min to waste
3.0 min to 8.5 min to MS
8.5 min to 11.0 min to waste

Injection volume: 25 µL

Retention time:

Prothioconazole- α -hydroxy-desthio: about 4.5 min
Prothioconazole-3-hydroxy-desthio: about. 6.2 min
Prothioconazole-4-hydroxy-desthio: about. 7.0 min
Prothioconazole-5-hydroxy-desthio: about. 7.3 min
Prothioconazole-6-hydroxy-desthio: about. 8.0 min

MS conditions

System: API 4000, SCIEX

Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)

Polarity: Positive ion mode

Scan type: MS/MS, Multiple reaction monitoring (MRM)

Ionspray turbo heater: 600°C

Capillary voltage (IS) 5500 V

Mass transitions:

Prothioconazole- α -hydroxy-desthio: 328 \rightarrow 70 m/z proposed for quantification and 328 \rightarrow 141 m/z proposed for confirmation
Prothioconazole-3-hydroxy-desthio: 328 \rightarrow 70 m/z proposed for quantification and 328 \rightarrow 141 m/z proposed for confirmation
Prothioconazole-4-hydroxy-desthio: 328 \rightarrow 70 m/z proposed for quantification and 328 \rightarrow 141 m/z proposed for confirmation
Prothioconazole-5-hydroxy-desthio: 328 \rightarrow 70 m/z proposed for quantification and 328 \rightarrow 141 m/z proposed for confirmation
Prothioconazole-6-hydroxy-desthio: 328 \rightarrow 70 m/z proposed for quantification and 328 \rightarrow 141 m/z proposed for confirmation

Analytical conditions for Triazole metabolites

LC conditions (Quantification)

System: Series 1290 HPLC, Agilent Technologies

Pre column: Phenomenex SecurityGuardTM for C18 HPLC

Column: Thermo Hypercarb, 100 mm x 3.0 mm, 5µm

Column temperature: 60°C

Flow: 0.6 mL/min

Mobile phase

Eluent A: Methanol + 0.5% v/v formic acid

Eluent B: Water + 0.5% v/v formic acid

Gradient:

Time (min)	% A	% B
0.00	0	100
3.00	0	100
6.50	80	20
6.51	0	100
9.00	0	100

Divert valve:

0.0 min to 0.7 min to waste
0.7 min to 6.5 min to MS

Injection volume: 20 µL

Retention time:

1,2,4-triazole: 1.4 min
Triazole alanine: 1.8 min

Triazole acetic acid: 4.8 min
Triazole lactic acid: 5.0 min

LC conditions (Confirmation)

System: Series 1290 HPLC, Agilent Technologies
Pre column: Phenomenex SecurityGuard™ for C18 HPLC
Column: Synergi 4µ Polar-RP 80A, 150 mm x 4.6 mm, 4µm
Column temperature: 40°C
Flow: 0.8 mL/min
Mobile phase
Eluent A: Methanol + 0.5% v/v formic acid
Eluent B: Water + 0.5% v/v formic acid
Gradient:

Time (min)	% A	% B
0.00	30	70
4.00	80	20
4.01	30	70
8.00	30	70

Divert valve:

1.0 min to 1.5 min to waste
1.3 min to 6.9 min to MS

Injection volume: 30 µL

Retention time:

1,2,4-triazole: 2.7 min
Triazole acetic acid: 2.7 min
Triazole lactic acid: 2.6 min

MS conditions

System: API TripleQuad 6500 system, SCIEX (Triple quadrupole mass spectrometer)
Ionisation type: Electrospray ionisation (ESI, TurbolonSpray)
Polarity: Positive ion mode
Scan type: MS/MS, Multiple reaction monitoring (MRM)
Ionspray turbo heater: 350°C
Capillary voltage (IS) 400 V
Mass transitions:
1,2,4-triazole: 70 → 43 m/z proposed for quantification
1,2,4-triazole (ISTD): 75 → 46 m/z
Triazole alanine: 157 → 70 m/z proposed for quantification, 157 → 88 m/z proposed for confirmation
Triazole alanine (ISTD): 162 → 75 m/z
Triazole acetic acid: 128 → 70 m/z proposed for quantification
Triazole acetic acid (ISTD): 133 → 75 m/z
Triazole lactic acid: 158 → 70 m/z proposed for quantification
Triazole acetic acid (ISTD): 163 → 75 m/z

Results and discussions

Table A 22: Recovery results from method validation of Prothioconazole-desthio using the analytical method

Prothioconazole-desthio							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 312 → 70 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	94, 99, 99	97	3.0	3	98	2.7
	0.1	98, 102, 97	99	2.7	3		

Wheat (grain)	0.01	81, 92, 99	91	10	3	93	7.2
	0.1	92, 98, 97	96	3.4	3		
Wheat (straw)	0.01	82, 112, 111, 101	102	14	4	104	12
	0.1	92, 117, 113	107	13	3		
Wheat (ears)	0.01	86, 85, 78	83	5.3	3	85	3.9
	0.1	86, 87, 85	86	1.2	3		
Wheat (rest of plant)	0.01	98, 100, 104, 86	97	8.0	4	99	6.3
	0.1	104, 102, 102	103	1.1	3		
	0.1	102, 103, 103, 107, 104	104	2	5		

Recoveries are without any blank correction

Table A 23: Recovery results from method validation of Prothioconazole- α -hydroxy-desthio using the analytical method

Prothioconazole- α -hydroxy-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 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	83, 102, 110, 111, 110, 109, 110	105	9.7	7	102	8.4
	0.1	106, 97, 96, 102, 103, 91	99	5.5	6		
Wheat (grain)	0.01	103, 90, 98	97	6.8	3	89	11
	0.1	79, 87, 79	82	5.7	3		
Wheat (straw)	0.01	95, 90, 105, 101	98	6.8	4	99	6.9
	0.1	102, 93, 109	101	7.9	3		
Wheat (ears)	0.01	113, 106, 105	108	4.0	3	106	4.5
	0.1	105, 106, 98	103	4.2	3		
Wheat (rest of plant)	0.01	107, 98, 103	103	4.4	3	101	3.6
	0.1	100, 100, 97	99	1.7	3		

Recoveries are without any blank correction

The LOQ is defined as PTZ-desthio.

Analyte: PTZ- α -OH-desthio

Final determination as: PTZ- α -OH-desthio

Residues calculated as: PTZ-desthio

Table A 24: Recovery results from method validation of Prothioconazole-3-hydroxy-desthio using the analytical method

Prothioconazole-3-hydroxy-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 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	71, 92, 93, 89, 92, 93, 94	89	9.1	7	93	9.9
	0.1	110, 94, 94, 96, 106, 86	98	9.0	6		
Wheat (grain)	0.01	100, 80, 82	87	13	3	89	8.8
	0.1	90, 85, 95	90	5.6	3		
Wheat (straw)	0.01	76, 80, 87, 90	83	7.7	4	89	11
	0.1	96, 89, 105	97	8.3	3		
Wheat (ears)	0.01	107, 102, 105	105	2.4	3	101	4.6
	0.1	100, 97, 95	97	2.6	3		
Wheat (rest of plant)	0.01	93, 83, 87	88	5.7	3	93	6.8
	0.1	97, 99, 96	97	1.6	3		

Recoveries are without any blank correction

The LOQ is defined as PTZ-desthio.

Analyte: PTZ-3-OH-desthio

Final determination as: PTZ-3-OH-desthio

Residues calculated as: PTZ-desthio

Table A 25: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical method

Prothioconazole-4-hydroxy-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 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	70, 90, 98, 96, 92, 83	89	11	7	93	9.8
	0.1	107, 97, 95, 101, 95, 87	97	6.9	6		
Wheat (grain)	0.01	102, 90, 88	93	8.1	3	92	6.4
	0.1	85, 91, 93	90	4.6	3		
Wheat (straw)	0.01	85, 80, 96, 94	89	8.5	4	88	7.4
	0.1	93, 82, 84	86	6.8	3		
Wheat (ears)	0.01	104, 105, 101	103	2.0	3	101	4.5
	0.1	105, 96, 95	99	5.6	3		
Wheat (rest of plant)	0.01	91, 92, 90	91	1.1	3	92	1.9
	0.1	92, 95, 91	93	2.2	3		

Recoveries are without any blank correction

The LOQ is defined as PTZ-desthio.

Analyte: PTZ-4-OH-desthio

Final determination as: PTZ-4-OH-desthio

Residues calculated as: PTZ-desthio

Table A 26: Recovery results from method validation of Prothioconazole-5-hydroxy-desthio using the analytical method

Prothioconazole-5-hydroxy-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 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	83, 83, 82, 82, 87, 80, 81	83	2.7	7	88	9.3
	0.1	108, 95, 90, 98, 95, 85	94	9.4	6		
Wheat (grain)	0.01	86, 80, 91	86	6.4	3	86	5.0
	0.1	82, 90, 87	86	4.7	3		
Wheat (straw)	0.01	94, 90, 94, 97	94	3.1	4	96	5.6
	0.1	97, 91, 106	98	7.7	3		
Wheat (ears)	0.01	107, 93, 81	94	14	3	94	10
	0.1	101, 95, 87	94	7.4	3		
Wheat (rest of plant)	0.01	104, 96, 104	101	4.6	3	99	4.4
	0.1	100, 99, 93	97	3.9	3		

Recoveries are without any blank correction

The LOQ is defined as PTZ-desthio.

Analyte: PTZ-5-OH-desthio

Final determination as: PTZ-5-OH-desthio

Residues calculated as: PTZ-desthio

Table A 27: Recovery results from method validation of Prothioconazole-6-hydroxy-desthio using the analytical method

Prothioconazole-6-hydroxy-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 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	70, 87, 90, 72, 84, 92, 96	84	12	7	85	10.4
	0.1	95, 91, 87, 83, 71, 89	86	9.7	6		
Wheat (grain)	0.01	85, 80, 83	83	3.0	3	83	3.5
	0.1	79, 85, 86	83	4.5	3		
Wheat (straw)	0.01	84, 80, 88	84	4.8	3	87	5.7
	0.1	90, 85, 94	90	5.0	3		

Wheat (ears)	0.01	102, 98, 98	99	2.3	3	90	8.0
	0.1	91, 84, 85	87	4.4	3		
Wheat (rest of plant)	0.01	86, 86, 85	86	0.7	3	84	3.2
	0.1	85, 85, 79	83	4.2	3		

Recoveries are without any blank correction

The LOQ is defined as PTZ-desthio.

Analyte: PTZ-6-OH-desthio

Final determination as: PTZ-6-OH-desthio

Residues calculated as: PTZ-desthio

Table A 28: Recovery results from method validation of 1,2,4-triazole 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 m/z 70 → 43 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	111, 115, 107, 107, 120, 107, 98	109	6.4	7	107	6.4
	0.1	93, 110, 103, 103, 109, 101, 109	104	5.8	7		
Wheat (grain)	0.01	102, 103, 112	106	5.2	3	103	6.5
	0.1	96, 108, 95	100	7.3	4		
Wheat (straw)	0.01	103, 99, 99, 98	100	2.2	4	103	4.8
	0.1	108, 103, 112, 100	106	5.0	4		
Wheat (ears)	0.01	114, 89, 114	106	14	3	105	9.3
	0.1	98, 104, 108	103	4.9	3		
Wheat (rest of plant)	0.01	88, 104, 114	102	13	3	106	11
	0.1	99, 119, 110	109	9.2	3		

Recoveries are corrected for the mean peak area of the control sample extract(s)

Analyte: 1,2,4-Triazole

Final determination as: 1,2,4-Triazole

Residues calculated as: 1,2,4-Triazole

Table A 29: Recovery results from method validation of Triazole Alanine using the analytical method

Triazole Alanine							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 157 → 70 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	119, 100, 97, 96, 95, 101, 85, 96	99	9.7	8	96	8.6
	0.1	88, 87, 97, 94, 92, 102, 89	93	5.8	7		
Wheat (grain)	0.01	80, 80, 83, 109	88	16	4	86	12
	0.1	83, 83, 85	84	1.4	3		
Wheat (straw)	0.01	71, 93, 82, 73, 84	81	11	5	82	8.8
	0.1	83, 87, 89, 78	84	5.8	4		
Wheat (ears)	0.01	91, 85, 72, 68	79	14	4	82	11
	0.1	92, 83, 86	87	5.3	3		
Wheat (rest of plant)	0.01	78, 90, 79	82	8.1	3	82	5.7
	0.1	80, 81, 86	82	3.9	3		

Recoveries are corrected for the mean peak area of the control sample extract(s)

Analyte: Triazole Alanine

Final determination as: Triazole Alanine

Residues calculated as: Triazole Alanine

Table A 30: Recovery results from method validation of Triazole acetic acid using the analytical method

Triazole acetic acid							
Matrix	Fortification Level	Recovery	Mean Recovery	Rel. Std. Dev.	Replicates	Overall Mean Recovery	Overall Rel. Std.

	(mg/kg)	(%)	(%)	(%)		(%)	Dev. (%)
Transition m/z 128 → 70 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	110, 98, 100, 104, 96, 102, 95, 86	99	7.2	8	100	5.5
	0.1	105, 98, 103, 102, 103, 100, 104	102	2.4	7		
Wheat (grain)	0.01	89, 82, 86, 86	86	3.3	4	85	2.7
	0.1	84, 87, 84	85	2.0	3		
Wheat (straw)	0.01	93, 93, 99, 101, 93	96	4.1	5	97	3.5
	0.1	96, 96, 102, 97	98	2.9	4		
Wheat (ears)	0.01	96, 89, 102, 78	91	11	4	92	8.4
	0.1	89, 97, 94	93	4.3	3		
Wheat (rest of plant)	0.01	87, 115, 105	102	14	3	101	9.6
	0.1	100, 95, 106	100	5.5	3		

Recoveries are corrected for the mean peak area of the control sample extract(s)

Analyte: Triazole acetic acid

Final determination as: Triazole acetic acid

Residues calculated as: Triazole acetic acid

Table A 31: Recovery results from method validation of Triazole lactic acid using the analytical method

Triazole lactic acid							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 158 → 70 m/z (Proposed for Quantification)							
Wheat (whole plant)	0.01	86, 72, 92, 94, 90, 89, 91, 95	89	8.2	8	93	8.9
	0.1	108, 106, 92, 93, 94, 98, 97	98	6.5	7		
Wheat (grain)	0.01	83, 79, 82, 70	79	7.5	4	76	7.1
	0.1	70, 76, 73	73	4.1	3		
Wheat (straw)	0.01	97, 84, 91, 93, 78	89	8.5	5	91	7.8
	0.1	103, 91, 90, 91	94	6.6	4		
Wheat (ears)	0.01	100, 91, 89, 73	88	13	4	91	10
	0.1	100, 89, 94	94	5.8	3		
Wheat (rest of plant)	0.01	95, 111, 107	104	8.0	3	105	6.4
	0.1	99, 112, 106	106	6.2	3		

Recoveries are corrected for the mean peak area of the control sample extract(s)

Analyte: Triazole lactic acid

Final determination as: Triazole lactic acid

Residues calculated as: Triazole lactic acid

Table A 32: Characteristics for the analytical method used for validation of Prothioconazole-desthio, prothioconazole metabolites and triazole derivate metabolites in wheat

	PTZ-desthio	PTZ metabolites	Triazole metabolites
Specificity	The analyte PTZ desthio was determined by use LC-MS/MS. One mass transition was evaluated. A second mass transition was monitored for confirmation peak identity. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention time of PTZ-desthio of the control sample materials that were used for determinations of the (procedural) recoveries did not exceed 30 % of the LOQ.	The analytes of PTZ metabolites were determined by use LC-MS/MS. For each analyte, one mass transition was evaluated. A second mass transition was monitored for confirmation peak identity. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention times of PTZ metabolites analytes of the control sample materials that were used for determinations of the (procedural) recoveries	The analytes of Triazole metabolites were determined by use LC-MS/MS. For each analyte one mass transition was evaluated. For each of the internal standards of 1,2,4-Triazole, TA, TAA and TLA one mass transition was evaluated. At least one control samples per matrix and analyte were extracted and analysed according to the method. The blank values at the expected retention time of 1,2,4-Triazole of the control sample materials did not exceed 30 % of the LOQ. The

	PTZ-desthio	PTZ metabolites	Triazole metabolites
		did not exceed 30 % of the LOQ.	blank values at the expected retention times of TA, TAA and TLA of the control sample materials did exceed 30 % of the LOQ. The situation was considered unavoidable. No other appropriate source of control samples of wheat could be found. Correction for blank values was performed even if they were below 30 % of the LOQ.
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A33 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A33 below.	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A33 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL ($n = 7$), corresponding to a range from 0.003 mg/kg to 0.2 mg/kg in sample extract which corresponds to more than 30% of the LOQ to + 20 % of the highest analyte concentration.	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL ($n = 7$), corresponding to a range from 0.003 mg/kg to 0.2 mg/kg in sample extract which corresponds to more than 30% of the LOQ to + 20 % of the highest analyte concentration.	Linearity was confirmed over the calibration range 0.30 – 400.0 ng/mL ($n = 7$), corresponding to a range from 0.003 mg/kg to 4.0 mg/kg in sample extract which corresponds to more than 30% of the LOQ to + 20 % of the highest analyte concentration.
Assessment of matrix effects is presented	Not in this study	Not in this study	No
Limit of determination/quantification	The LOQ is 0.01 mg/kg in all matrices. The limit of detection (LOD) is set at 30% of the LOQ which is 0.003 mg/kg.	The LOQ is 0.01 mg/kg for each analyte in all matrices. The limit of detection (LOD) is set at 30% of the LOQ which is 0.003 mg/kg.	The LOQ is 0.01 mg/kg for each analyte in all matrices. The limit of detection (LOD) is set at 30% of the LOQ which is 0.003 mg/kg.
Stability of standards and extracts	The stability of the analyte PTZ-desthio in the final extracts of wheat (grain) upon storage at typically 1 °C to 10 °C for nine days was demonstrated in S16-04434. For a high water matrix (cucumber) a storage stability for nine days was demonstrated	The stability of the analytes in the final extracts of wheat (whole plant) upon storage at typically 1 °C to 10 °C for 20 days was demonstrated in S16-04435. For wheat (grain) a storage stability for 17 days and for wheat (straw) a storage stability for 14 days was demonstrated.	Due to the use of internal standards the stability of the TDMs 1,2,4-Triazole, TA, TAA and TLA in the final extracts was not assessed in S15-03542.

Table A 33: Linearity of detector response

Analyte	Matrix/Transition	Calibration range	Equation	r
PTZ-desthio	Wheat (whole plant) m/z 312/70 (Quanti.)	0.15 – 10.0 ng/mL ($n = 7$)	$Y = 328561.5365x + 8247.8645$	0.9999
	Wheat (grain) m/z 312/70 (Quanti.)	0.15 – 10.0 ng/mL ($n = 7$)	$Y = 525351.0235x + 10242.1748$	0.9987
	Wheat (straw) m/z 312/70 (Quanti.)	0.15 – 10.0 ng/mL ($n = 7$)	$Y = 286782.4109x + 2964.1070$	0.9998
	Wheat (ears) m/z 312/70 (Quanti.)	0.15 – 10.0 ng/mL ($n = 7$)	$Y = 478177.9126x + 24294.0900$	0.9999

	Wheat (rest of plant) m/z 312/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 371343.9474 + 2583.4001$	0.9997
PTZ- α -hydroxy- desthio	Wheat (whole plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 24901.7437x + 3419.7899$	0.9988
	Wheat (grain) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 8765.6305x - 474.8648$	0.9983
	Wheat (straw) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 3577.1291x + 122.3574$	0.9982
	Wheat (ears) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 3431.4683x - 103.5185$	0.9957
	Wheat (rest of plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 3556.5782x - 29.9728$	0.9988
PTZ-3-hydroxy- desthio	Wheat (whole plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 31121.6578x + 5701.1170$	0.9981
	Wheat (grain) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 10933.6871x + 201.0340$	0.9996
	Wheat (straw) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 3941.3748x + 338.7660$	0.9994
	Wheat (ears) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 5365.5598x + 4.5475$	0.9990
	Wheat (rest of plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 4295.4865x + 177.6122$	0.9996
PTZ-4-hydroxy- desthio	Wheat (whole plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 33463.9373x + 7598.0100$	0.9977
	Wheat (grain) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 12325.5497x + 356.3002$	0.9998
	Wheat (straw) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 3796.9573x + 329.0126$	0.9987
	Wheat (ears) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 5634.7890x + 117.1248$	0.9981
	Wheat (rest of plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 4744.4832x + 111.9318$	0.9999
PTZ-5-hydroxy- desthio	Wheat (whole plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 28265.7569x + 6276.7970$	0.9963
	Wheat (grain) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 11623.8059x - 46.3468$	0.9999
	Wheat (straw) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 4535.1168x + 142.9698$	0.9996
	Wheat (ears) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 5900.1264x + 165.9475$	0.9995
	Wheat (rest of plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 4445.0341x + 81.4058$	0.9999
PTZ-6-hydroxy- desthio	Wheat (whole plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 32262.4210x + 5532.3542$	0.9977
	Wheat (grain) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 11632.2467x - 85.6248$	0.9997
	Wheat (straw) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 5053.1716x + 26.1396$	0.9999
	Wheat (ears) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 6166.4089x - 31.2567$	0.9980
	Wheat (rest of plant) m/z 328/70 (Quanti.)	0.15 – 10.0 ng/mL (n = 7)	$Y = 5517.2302x - 45.4551$	0.9999
1,2,4-Triazole	Wheat (whole plant) m/z 70/43 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1013x - 0.0078$	0.9975
	Wheat (grain) m/z 70/43 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.0810x - 0.0006$	0.9997
	Wheat (straw) m/z 70/43 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.0907x + 0.0054$	0.9970
	Wheat (rest of plant) m/z 70/43 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1046x - 0.0057$	0.9992
	Wheat (ears) m/z 70/43 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1055x + 0.0043$	0.9997
Triazole Alanine	Wheat (whole plant) m/z 157/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1562x - 0.0209$	0.9952
	Wheat (grain) m/z 157/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1290x + 0.0034$	0.9998

	Wheat (straw) m/z 157/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1523x - 0.0028$	0.9997
	Wheat (rest of plant) m/z 157/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1671 - 0.0048$	0.9982
	Wheat (ears) m/z 157/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1581x - 0.0022$	0.9982
Triazole acetic acid	Wheat (whole plant) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1379x - 0.0103$	0.9987
	Wheat (grain) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1088x - 0.0032$	0.9999
	Wheat (straw) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1396x + 0.0065$	0.9994
	Wheat (rest of plant) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1394x - 0.0094$	0.9997
	Wheat (ears) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.1641x + 0.0051$	0.9998
Triazole lactic acid	Wheat (whole plant) m/z 158/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.3082x + 0.0552$	0.9959
	Wheat (grain) m/z 158/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.2290x - 0.0112$	0.9998
	Wheat (straw) m/z 158/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.3306x - 0.0079$	0.9988
	Wheat (rest of plant) m/z 158/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.2345x - 0.0014$	0.9999
	Wheat (ears) m/z 128/70 (Quanti.)	0.30 – 400.0 ng/mL (n = 10)	$Y = 0.2381x - 0.0004$	0.9998

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1 for the determination of prothioconazole-desthio, prothioconazole-alpha-hydroxy-desthio, -3-hydroxyl-desthio, -4-hydroxy-desthio, -5-hydroxy-desthio and -6-hydroxy-desthio, as well as 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in wheat matrices. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for all analytes in all matrices.

A 2.1.1.1.5.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.5.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.6 Prothioconazole metabolites

A 2.1.1.1.6.1 Method validation 1

Comments of zRMS:	The analytical methods 00979 and 00979/M001 were successfully validated for the determination of residues of JAU 6476-a-hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio, and JAU 6476-6-hydroxy-desthio in/on matrices of plant origin and are acceptable.
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Reference:	KCP 5.1.2/08
Report	Analytical Method 00979 for the determination of residues of JAU 6476 3 hydroxy desthio, JAU 6476 4 hydroxy desthio, JAU 6476 5 hydroxy desthio, and JAU 6476 6 hydroxy desthio in/on Matrices of Plant Origin by HPLC-MS/MS, Freitag T, 2006, Report No. M-267072-01-1
Guideline(s):	SANCO/3029/99
Deviations:	Not specified
GLP:	Yes
Acceptability:	Yes
Reference:	KCP 5.1.2/09
Report	Analytical Method 00979/M001 for the determination of residues of JAU 6476- α -hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio, and JAU 6476-6-hydroxy-desthio in/on matrices of plant origin by HPLC-MS/MS, Freitag Th. and Daniels M., 2009, Report No. M-328686-01-1
Guideline(s):	SANCO/3029/99
Deviations:	Not specified
GLP:	Yes
Acceptability:	Yes

Materials and methods

The homogenised sample material of plant origin is extracted with a mixture of acetonitrile/water 4:1 (v:v) by high-speed blending. After filtration and evaporation to the aqueous remainder the extract is diluted and acidified with 5 N hydrochloric acid and refluxed for 2 hours. This hydrolysis step is performed to convert glycoside-bound analogues into the respective hydroxy analytes. An aliquot is neutralised with sodium hydrogen carbonate and purified on a Chromabond® XTR cartridge. The analytes are eluted with cyclohexane/ethyl acetate 85:15 (v:v). The eluate was evaporated to dryness and the remainder is resolved in acetonitrile. For quantitative analysis, the extract is diluted with acetonitrile and water and subjected to HPLC-MS/MS. All analytes are detected using electrospray ionization in the positive ion mode (ESI+). For quantification external calibration with matrix matched standard solutions is applied. Results are expressed as JAU 6476-desthio.

MRM mass transitions for quantification and confirmation of JAU 6476- α -hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio and JAU 6476-6-hydroxy-desthio:

Analyte	Transition mass	
	for quantification	for confirmation
JAU 6476- α -hydroxy-desthio	m/z 328 \rightarrow 70	m/z 328 \rightarrow 141
JAU 6476-3-hydroxy-desthio	m/z 328 \rightarrow 70	m/z 328 \rightarrow 141
JAU 6476-4-hydroxy-desthio	m/z 328 \rightarrow 70	m/z 328 \rightarrow 141
JAU 6476-5-hydroxy-desthio	m/z 328 \rightarrow 70	m/z 328 \rightarrow 141
JAU 6476-6-hydroxy-desthio	m/z 328 \rightarrow 70	m/z 328 \rightarrow 141

Results and discussions

Table A 34: Recovery results from method validation of Prothioconazole- α -hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole- α -hydroxy- desthio							
Matrix	Fortification Level	Recovery	Mean Recovery	Rel. Std.	Replicates	Overall Mean	Overall Rel.

	(mg/kg)	(%)	(%)	Dev. (%)		Recovery (%)	Std. Dev. (%)
Transition m/z 328 → 70 m/z (Proposed for Quantification)							
Wheat grain*	0.01	93 , 96 , 92 , 93 , 97	94	2.3	5	95	2.5
	0.20	92 , 96 , 92 , 98 , 96	95	2.8	5		
Potato tuber*	0.01	77, 89, 86, 185 ^s , 91	86	7.2	4	89	6.3
	0.20	85, 92, 88, 96, 93	91	4.8	5		
Tomato fruit*	0.01	89, 95, 94, 94, 95	93	2.7	5	91	3.3
	0.20	91, 88, 88, 92, 88	89	2.2	5		
Rape seed*	0.01	81, 102, 101, 92, 103	96	9.8	5	97	6.6
	0.20	97, 98, 98, 95, 100	98	1.9	5		
Orange fruit*	0.01	100, 102, 99, 101, 99	100	2.3	5	96	5.4
	0.20	88, 91, 93, 92, 90	91	2.1	5		

* Validation of method M-328686-01-1

** Validation of method M-267072-01-1

Table A 35: Recovery results from method validation of Prothioconazole-3-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-3-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	95 , 92 , 87 , 97 , 99	94	5.0	5	95	3.7
	0.20	94, 96, 94, 98, 97	96	1.9	5		
Wheat grain**	0.01	81, 99, 84, 83, 82	86	8.7	5	88	6.4
	0.20	88, 85, 90, 91, 92	89	3.1	5		
Potato tuber*	0.01	85, 86, 85, 189 ^s , 91	87	3.3	4	89	4.8
	0.20	86, 93, 88, 97, 93	91	4.8	5		
Tomato fruit*	0.01	85, 95, 89, 92, 91	90	4.1	5	89	4.5
	0.20	91, 85, 84, 92, 84	87	4.5	5		
Rape seed*	0.01	81, 94, 95, 91, 96	91	6.7	5	95	6.0
	0.20	98, 99, 99, 95, 101	98	2.2	5		
Orange fruit*	0.01	95, 93, 95, 99, 94	95	2.4	5	92	4.1
	0.20	86, 90, 92, 91, 88	89	2.7	5		

* Validation of method M-328686-01-1

** Validation of method M-267072-01-1

Table A 36: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical method

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-4-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	91, 91, 89, 95, 96	92	3.2	5	94	2.9
	0.20	93, 95, 93, 98, 95	95	2.2	5		
Wheat grain**	0.01	81, 102 , 83, 82, 80	86	10.8	5	87	7.7
	0.20	88, 83, 89, 90, 92	88	3.8	5		
Potato tuber*	0.01	82, 82, 78, 184 ^s , 87	82	4.5	4	86	6.0
	0.20	85, 89, 86, 95, 91	89	4.5	5		
Tomato fruit*	0.01	89, 96, 91, 91, 92	92	2.8	5	89	4.3
	0.20	90, 85, 84, 90, 84	87	3.6	5		
Rape seed*	0.01	83, 92, 95, 84, 97	90	7.1	5	94	6.2
	0.20	98, 98, 96, 93, 100	97	2.7	5		
Orange fruit*	0.01	96, 94, 93, 97, 96	95	1.7	5	92	3.9
	0.20	87, 90, 92, 91, 87	89	2.6	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 37: Recovery results from method validation of Prothioconazole-5-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-5-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	97, 96, 92, 95, 99	96	2.7	5	96	2.1
	0.20	94, 97, 95, 98, 96	96	1.6	5		
Wheat grain**	0.01	88, 91, 94, 89, 88	90	2.8	5	90	3.2
	0.20	88, 84, 90, 91, 93	89	3.8	5		
Potato tuber*	0.01	82, 82, 82, 186 [§] , 87	83	3.0	4	87	5.2
	0.20	85, 90, 88, 95, 90	90	4.1	5		
Tomato fruit*	0.01	92, 97, 92, 92, 95	94	2.5	5	91	4.4
	0.20	90, 86, 85, 91, 86	88	3.1	5		
Rape seed*	0.01	84, 94, 98, 89, 99	93	6.8	5	94	5.0
	0.20	95, 96, 96, 94, 99	96	1.9	5		
Orange fruit*	0.01	96, 92, 95, 102, 101	97	4.3	5	94	5.4
	0.20	87, 90, 92, 92, 88	90	2.5	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 38: Recovery results from method validation of Prothioconazole-6-hydroxy-desthio using the analytical method

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-6-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	97, 93, 92, 99, 94	95	3.1	5	96	2.7
	0.20	94, 96, 94, 98, 99	96	2.4	5		
Wheat grain**	0.01	92, 101, 90, 86, 87	91	6.6	5	90	6.2
	0.20	86, 81, 87, 92, 94	88	5.8	5		
Potato tuber*	0.01	107, 103, 103, 232 [§] , 109	106	2.8	4	113	7.1
	0.20	113, 122, 113, 124, 121	119	4.4	5		
Tomato fruit*	0.01	90, 102, 91, 96, 96	95	5.0	5	90	7.7
	0.20	88, 81, 83, 89, 81	84	4.6	5		
Rape seed*	0.01	74, 101, 99, 85, 109	94	14.9	5	95	11.5
	0.20	103, 99, 93, 83, 102	96	8.6	5		
Orange fruit*	0.01	98, 101, 97, 98, 110	101	5.3	5	96	6.8
	0.20	88, 91, 92, 93, 90	91	2.1	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 39: Recovery results from method validation of Prothioconazole- α -hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole- α -hydroxy-desthio							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)

Transition m/z 328 → 141 m/z (Proposed for Quantification)							
Wheat grain*	0.01	98, 95, 92, 101, 93	96	3.9	5	96	2.9
	0.20	95, 96, 93, 98, 97	96	2.0	5		
Potato tuber*	0.01	78, 85, 80, 176 [§] , 90	83	6.5	4	87	6.8
	0.20	86, 92, 87, 95, 94	91	4.5	5		
Tomato fruit*	0.01	91, 95, 87, 92, 96	92	3.9	5	91	3.9
	0.20	91, 88, 88, 92, 88	89	2.2	5		
Rape seed*	0.01	82, 101, 94, 92, 105	95	9.4	5	96	6.6
	0.20	97, 99, 97, 94, 101	98	2.7	5		
Orange fruit*	0.01	98, 102, 91, 100, 94	97	4.6	5	94	5.1
	0.20	88, 90, 93, 91, 90	90	2.0	5		
Wheat grain*	0.01	98, 95, 92, 101, 93	96	3.9	5	96	2.9
	0.20	95, 96, 93, 98, 97	96	2.0	5		

* Validation of method M-328686-01-1

** Validation of method M-267072-01-1

Table A 40: Recovery results from method validation of Prothioconazole-3-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-3-hydroxy-desthio							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 328 → 141 m/z (Proposed for Quantification)							
Wheat grain*	0.01	96, 94, 85, 91, 97	93	5.2	5	94	4.1
	0.20	94, 96, 94, 97, 98	96	1.9	5		
Wheat grain**	0.01	84, 102, 83, 82, 83	87	9.8	5	88	6.9
	0.20	88, 85, 90, 91, 92	89	3.1	5		
Potato tuber*	0.01	86, 83, 83, 182 [§] , 94	87	6.0	4	88	5.3
	0.20	84, 91, 86, 95, 91	89	4.9	5		
Tomato fruit*	0.01	84, 97, 89, 92, 93	91	5.3	5	89	5.1
	0.20	90, 85, 84, 90, 84	87	3.6	5		
Rape seed*	0.01	84, 100, 94, 89, 102	94	8.0	5	96	5.9
	0.20	97, 99, 98, 95, 101	98	2.3	5		
Orange fruit*	0.01	97, 100, 98, 102, 90	97	4.7	5	93	5.8
	0.20	86, 90, 92, 91, 88	89	2.7	5		

* Validation of method M-328686-01-1

** Validation of method M-267072-01-1

Table A 41: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-4-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	94, 91, 92, 93, 92	92	1.2	5	94	2.0
	0.20	93, 95, 93, 97, 96	95	1.9	5		
Wheat grain**	0.01	79, 99, 81, 79, 80	84	10.3	5	86	7.7
	0.20	88, 84, 89, 90, 92	89	3.3	5		
Potato tuber*	0.01	83, 82, 82, 186 [§] , 88	84	3.4	4	87	5.3
	0.20	86, 91, 86, 95, 92	90	4.4	5		
Tomato fruit*	0.01	86, 94, 90, 91, 92	91	3.3	5	89	4.0
	0.20	90, 85, 84, 90, 84	87	3.6	5		
Rape seed*	0.01	83, 95, 97, 90, 100	93	7.2	5	95	5.7
	0.20	99, 98, 97, 93, 101	98	3.0	5		
Orange fruit*	0.01	95, 91, 95, 96, 93	94	2.1	5	92	3.5
	0.20	87, 89, 92, 91, 87	89	2.6	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 42: Recovery results from method validation of Prothioconazole-5-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-5-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	94, 95, 88, 96, 98	94	4.0	5	95	3.1
	0.20	96, 97, 95, 98, 97	97	1.2	5		
Wheat grain**	0.01	89, 89, 93, 87, 94	90	3.3	5	90	3.2
	0.20	88, 85, 89, 91, 93	89	3.4	5		
Potato tuber*	0.01	82, 85, 83, 183 [§] , 95	86	6.9	4	88	5.6
	0.20	86, 91, 87, 95, 92	90	4.1	5		
Tomato fruit*	0.01	86, 95, 91, 90, 95	91	4.1	5	89	4.3
	0.20	90, 86, 85, 91, 85	87	3.3	5		
Rape seed*	0.01	89, 98, 101, 89, 96	95	5.7	5	96	4.1
	0.20	97, 97, 96, 94, 99	97	1.9	5		
Orange fruit*	0.01	97, 92, 93, 97, 97	95	2.6	5	92	4.0
	0.20	87, 90, 92, 91, 88	90	2.3	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 43: Recovery results from method validation of Prothioconazole-6-hydroxy-desthio using the analytical methods

Recoveries calculated via matrix standard; residues are expressed as JAU 6476-desthio

Prothioconazole-6-hydroxy-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 m/z (Proposed for Quantification)							
Wheat grain*	0.01	96, 98, 95, 96, 106	98	4.6	5	98	3.7
	0.20	95, 98, 93, 99, 100	97	3.0	5		
Wheat grain**	0.01	91, 101, 90, 88, 88	92	5.9	5	89	5.9
	0.20	84, 81, 87, 90, 91	87	4.8	5		
Potato tuber*	0.01	112, 102, 105, 268 [§] , 114	108	5.2	4	113	7.2
	0.20	112, 122, 110, 121, 117	116	4.6	5		
Tomato fruit*	0.01	86, 98, 91, 87, 94	91	5.4	5	88	6.5
	0.20	87, 80, 83, 89, 81	84	4.6	5		
Rape seed*	0.01	88, 92, 98, 91, 111	96	9.5	5	96	8.3
	0.20	102, 99, 92, 84, 102	96	8.1	5		
Orange fruit*	0.01	98, 95, 85, 95, 110	97	9.3	5	94	7.2
	0.20	89, 92, 91, 94, 90	91	2.1	5		

* Validation of method M-328686-01-1
** Validation of method M-267072-01-1

Table A 44: Characteristics for the analytical method used for validation of prothioconazole metabolites

	PTZ metabolites
Specificity	No apparent residues (<30% of LOQ) were detected in any of the corresponding control samples for all analytes and all matrices. For confirmation of the individual residues a second MRM transition was used for each analyte.
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 performed with 1/x weighting regression (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 .

	PTZ metabolites
Calibration range	Linearity was confirmed over the calibration range 0.01 – 10.0 µg/L (n = 18)
Assessment of matrix effects is presented	Not specified
Limit of determination/quantification	The LOQ is 0.01 mg/kg (expressed as prothioconazole-desthio equivalent) for each analyte in all matrices tested.
Stability of standards and extracts	The stability in extracts was tested in study M328686-01-1 for all matrices and analytes for a period of one and two days of storage in a refrigerator at about 4 °C ± 3 °C. All items were found to be stable for two days in all final extracts.

Conclusion

The two methods were successfully validated for the determination of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole -5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in/on plant matrices (wheat grain, potato tuber, tomato fruit, oilseed rape seed, orange fruit). The results of the method validation were confirmed using a second MRM transition. A quantification limit of 0.01 mg/kg was achieved for the determination of all analytes in all matrices tested. The above data are in line with the requirements outlined in the SANTE/2020/12830, Rev.2.

A 2.1.1.1.6.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.6.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.6.4 Method validation 2

Comments of zRMS:	The modification M002 of the analytical method 00979 is acceptable.
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Reference: KCP 5.1.2/10

Report Modification M002 of the analytical method 00979/M001 for the determination of the metabolites JAU 6476- α -hydroxy-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio and JAU 6476-6-hydroxy-desthio in plant matrices by HPLC-MS/MS, Glaubitz, J.; Hennes, M., 2016, Report No. M-513336-02-1

Guideline(s): SANCO/3029/99

Deviations: Not specified

GLP: Yes

Acceptability: Yes

Materials and methods

The modification M002 of the analytical method 00979 was developed and validated for the determination of residues of JAU 6476- α -hydroxy-desthio, JAU 6476-3-hydroxydesthio, JAU 6476-4-hydroxy-desthio, JAU 6476-5-hydroxy-desthio and JAU 6476-6-hydroxy-desthio in/on plant matrices by HPLC-MS/MS. This modification was made necessary, as stable-isotope labeled internal standards became available and the method had to be adapted accordingly. In the course of this modification also the extraction conditions are changed from macerating to shaking with an overhead shaker. The extraction efficiency was also tested in this study with incurred samples from previous studies.

2.5 g of the sample was extracted with 40 mL of a mixture of 4:1 (v/v) acetonitrile/water by shaking for 20 min. After adding the internal standard, the solution was shaken briefly and centrifuged. A 10 mL aliquot of this crude extract was evaporated to its aqueous remainder, which was then acidified with 5 N hydrochloric acid and refluxed for 2 hours. This hydrolysis step was performed to convert glycosidic bound analogues into the hydroxyl analytes. The culture tube is then rinsed with 5 mL of water followed by 5 mL of methanol, which are collected in a volumetric flask. The solution is then neutralized with ammonium carbonate and then subjected to analysis via HPLC-MS/MS. Detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in positive ion mode and multiple reaction monitoring (MRM). The conditions on the mass spectrometer are the same as in modification M001 of method 00979.

Results and discussions

Table A 45: Recovery results from method validation of Prothioconazole- α -hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
Alpha-hydroxy-prothioconazole-desthio (Ultra Turrax)	Orange, fruit	0.01	6	90	9.0
		0.10	6	93	5.5
	Bean, seed	0.01	6	101	9.0
		0.10	6	90	11.89
	Rape, seed**	0.01	9	97	9.1
		0.10	9	92	11.9
	Cereal, grain**	0.01	8	90	11.4
		0.10	8	90	10.7
	Strawberry, fruit	0.01	6	96	13.8
		0.10	6	97	6.1
	Barley, green material**, b	0.01	4	103	8.0
		0.20	4	96	5.3
	Wheat, straw**, b	0.01	4	80	21.3 ^a
		0.50	4	86	10.7

* expressed as prothioconazole-desthio

** These results were obtained as concurrent recoveries during the conduct of the cross validation of the extraction method using Ultra Turrax compared to the extraction method using an overhead shaker. Cereal grain comprises wheat and barley

a This RSD will be accepted, because it is only slightly above 20%.

b Reduced validation set.

Table A 46: Recovery results from method validation of Prothioconazole- α -hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
Alpha-hydroxy- prothioconazole-desthio (Overhead shaker)	Orange, fruit	0.01	5	105	2.6
		0.10	5	102	6.6
	Bean, seed	0.01	7	102	6.9
		0.10	7	97	12.3
	Rape, seed	0.01	7	107	6.8
		0.10	7	94	11.4
	Wheat, grain	0.01	5	97	4.0
		0.10	5	105	5.4
	Strawberry, fruit	0.01	6	100	9.1
		0.10	6	100	14.8
	Barley, green material**	0.01	4	101	10.6
		0.20	4	104	16.9
	Wheat, straw**	0.01	4	111 ^a	7.8
		0.50	4	102	8.8

* expressed as prothioconazole-desthio

** Reduced validation set

^a This recovery is acceptable, because it is only slightly above 110%.

Table A 47: Recovery results from method validation of Prothioconazole-3-hydroxy-desthio using the analytical method

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
3-hydroxy- prothioconazole- desthio (Ultra Turrax)	Orange, fruit	0.01	6	92	12.1
		0.10	6	100	7.8
	Bean, seed	0.01	6	90	10.0
		0.10	6	88	5.3
	Rape, seed**	0.01	9	102	11.3
		0.10	9	91	6.7
	Cereal, grain**	0.01	8	93	12.0
		0.10	8	82	12.5
	Strawberry, fruit	0.01	6	101	7.5
		0.10	6	89	3.8
	Barley, green material**, a	0.01	4	95	9.9
		0.20	4	94	3.6
	Wheat, straw**, a	0.01	4	76	10.0
		0.50	4	92	3.2

* expressed as prothioconazole-desthio

** Reduced validation set a These results were obtained as concurrent recoveries during the conduct of the cross validation of the extraction method using Ultra Turrax compared to the extraction method using an overhead shaker. Cereal grain comprises wheat and barley

Table A 48: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
3-hydroxy- prothioconazole- desthio (Overhead shaker)	Orange, fruit	0.01	5	101	6.4
		0.10	5	107	4.7
	Bean, seed	0.01	7	100	11.5
		0.10	6	98	11.4
	Rape, seed	0.01	7	98	10.1
		0.10	7	100	10.3
	Wheat, grain	0.01	5	97	8.6
		0.10	5	110	4.9
	Strawberry, fruit	0.01	6	103	13.0
		0.10	6	106	10.5
	Barley, green material**	0.01	4	98	10.1
		0.20	4	114 ^a	1.9
	Wheat, straw**	0.01	4	91	7.2
		0.50	4	104	7.1

* expressed as prothioconazole-desthio ** Reduced validation set ^aThis recovery will be accepted, because it is only slightly above 110%. FL: fortification level.

Table A 49: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical method

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
4-hydroxy- prothioconazole-desthio (Ultra Turrax)	Orange, fruit	0.01	6	75	14.1
		0.10	6	101	16.0
	Bean, seed	0.01	6	71	5.9
		0.10	6	78	6.9
	Rape, seed**	0.01	9	101	11.6
		0.10	9	90	15.7
	Cereal, grain**	0.01	8	87	13.9
		0.10	8	86	9.9
	Strawberry, fruit	0.01	6	82	16.2
		0.10	6	100	15.7
	Barley, green material** b	0.01	4	99	15.3
		0.20	4	110	13.4
	Wheat, straw**	0.01	3	89	16.2
		0.50	4	89	8.8

* expressed as prothioconazole-desthio

** Reduced validation set .These results were obtained as concurrent recoveries during the conduct of the cross validation of the extraction method using Ultra Turrax compared to the extraction method using an overhead shaker. Cereal grain comprises wheat and barley FL: fortification level

Table A 50: Recovery results from method validation of Prothioconazole-4-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
4-hydroxy- prothioconazole-desthio (Overhead shaker)	Orange, fruit	0.01	5	102	3.8
		0.10	5	104	3.3
	Bean, seed	0.01	7	114 ^a	11.0
		0.10	7	98	13.2
	Rape, seed	0.01	7	100	8.0
		0.10	7	106	10.9
	Wheat, grain	0.01	5	94	5.8
		0.10	5	86	8.9
	Strawberry, fruit	0.01	6	95	16.6
		0.10	6	109	11.4
	Barley, green material**	0.01	4	97	9.7
		0.20	4	102	13.3
	Wheat, straw**	0.01	4	104	9.0
		0.50	4	105	8.2

* expressed as prothioconazole-desthio

** Reduced validation set. ^a This recovery is acceptable, because it is only slightly above 110%.

FL: fortification level

Table A 51: Recovery results from method validation of Prothioconazole-5-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
5-hydroxy- prothioconazole-desthio (Ultra Turrax)	Orange, fruit	0.01	6	94	9.5
		0.10	6	94	11.5
	Bean, seed	0.01	6	93	7.5
		0.10	6	97	7.7
	Rape, seed**	0.01	9	100	14.1
		0.10	9	86	16.2
	Cereal, grain**	0.01	8	93	17.3
		0.10	8	88	11.6
	Strawberry, fruit	0.01	6	92	6.9
		0.10	6	100	9.2
	Barley, green material**, ^a	0.01	4	94	10.4
		0.20	4	96	10.3
	Wheat, straw**, ^a	0.01	3	73	9.3
		0.50	4	99	9.3

* expressed as prothioconazole-desthio

** ^a Reduced validation set. These results were obtained as concurrent recoveries during the conduct of the cross validation of the extraction method using Ultra Turrax compared to the extraction method using an overhead shaker. Cereal grain comprises wheat and barley FL: fortification level

Table A 52: Recovery results from method validation of Prothioconazole-5-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
5-hydroxy- prothioconazole-desthio (Overhead shaker)	Orange, fruit	0.01	5	98	8.3
		0.10	5	100	5.6
	Bean, seed	0.01	7	94	8.3
		0.10	7	94	10.5
	Rape, seed	0.01	7	100	9.8
		0.10	7	91	9.0
	Wheat, grain	0.01	5	94	8.0
		0.10	5	100	12.3
	Strawberry, fruit	0.01	6	97	14.0
		0.10	6	92	6.3
	Barley, green material**	0.01	4	98	11.0
		0.20	4	98	11.0
	Wheat, straw**	0.01	4	109	6.4
		0.50	4	100	13.7

* expressed as prothioconazole-desthio ** Reduced validation set. FL: fortification level

Table A 53: Recovery results from method validation of Prothioconazole-6-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
6-hydroxy- prothioconazole-desthio (Ultra Turrax)	Orange, fruit	0.01	6	99	8.9
		0.10	6	101	6.0
	Bean, seed	0.01	6	81	12.2
		0.10	6	88	9.5
	Rape, seed**	0.01	9	103	11.2
		0.10	9	100	11.9
	Cereal, grain**	0.01	8	93	9.9
		0.10	8	87	14.1
	Strawberry, fruit	0.01	6	80	11.8
		0.10	6	93	9.0
	Barley, green material**, a	0.01	4	107	2.2
		0.20	4	90	10.3
	Wheat, straw**, a	0.01	3	83	13.7
		0.50	4	81	15.1

* expressed as prothioconazole-desthio

** Reduced validation set. ^aThese results were obtained as concurrent recoveries during the conduct of the cross validation of the extraction method using Ultra Turrax compared to the extraction method using an overhead shaker. Cereal grain comprises wheat and barley FL: fortification level

Table A 54: Recovery results from method validation of Prothioconazole-6-hydroxy-desthio using the analytical methods

Analyte	Matrix	FL [mg/kg]*	n	Mean [%]	RSD [%]
6-hydroxy-prothioconazole-desthio (Overhead shaker)	Orange, fruit	0.01	5	101	6.4
		0.10	5	99	5.8
	Bean, seed	0.01	7	101	12.6
		0.10	7	92	11.9
	Rape, seed	0.01	7	97	9.6
		0.10	7	94	7.2
	Wheat, grain	0.01	5	103	9.0
		0.10	5	91	5.0
	Strawberry, fruit	0.01	6	95	13.7
		0.10	6	100	10.3
	Barley, green material**	0.01	4	102	3.8
		0.20	4	88	9.6
	Wheat, straw**	0.01	4	116 ^a	14.1
		0.50	4	100	10.9

* expressed as prothioconazole-desthio ** Reduced validation set. ^aThis recovery is acceptable, because it is only slightly above 110%.

FL: fortification level

Table A 55: Characteristics for the analytical method used for validation of prothioconazole-desthio residue – Ultra Turrax

Ultra Turrax	prothioconazole- α -hydroxy-desthio	Prothioconazole-3-hydroxy-desthio	Prothioconazole-4-hydroxy-desthio
Specificity	mass spectra provided in Appendix 5 of the report blank value < 30 % LOQ)	mass spectra provided in Appendix 5 of the report blank value < 30 % LOQ)	mass spectra provided in Appendix 5 of the report blank value < 30 % LOQ)
Calibration (type, number of data points)	Calibration data presented calibration line equation presented number of data points ≥ 5 always > 0.99	Calibration data presented calibration line equation presented number of data points ≥ 5 always > 0.99	Calibration data presented calibration line equation presented number of data points ≥ 5 always > 0.99
Calibration range	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)
Assessment of matrix effects is presented	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.
Limit of determination/quantification	LOQ: 0.01 mg/kg Calculated LOD: 0.003 to 0.004 mg/kg depending on matrix	LOQ: 0.01 mg/kg Calculated LOD: 0.003 to 0.004 mg/kg depending on matrix	LOQ: 0.01 mg/kg Calculated LOD: 0.001 to 0.005 mg/kg depending on matrix

Ultra Turrax	prothioconazole-5-hydroxy-desthio	Prothioconazole-6-hydroxy-desthio
Specificity	mass spectra provided in Appendix 5 of the report blank value < 30 % LOQ)	mass spectra provided in Appendix 5 of the report blank value < 30 % LOQ)
Calibration (type, number of data points)	Calibration data presented calibration line equation presented number of data points ≥ 5 always > 0.99	Calibration data presented calibration line equation presented number of data points ≥ 5 always > 0.99
Calibration range	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 µg/L (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 µg/L (corresponding to 0.005 to 1.0 mg/kg)
Assessment of matrix effects is presented	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.
Limit of determination/quantification	LOQ: 0.01 mg/kg Calculated LOD: 0.002 to 0.006 mg/kg depending on matrix	LOQ: 0.01 mg/kg Calculated LOD: 0.003 mg/kg for all matrices

Table A 56: Characteristics for the analytical method used for validation of prothioconazole-desthio residue – Overhead shaker

Overhead shaker	Prothioconazole- α -desthio	Prothioconazole -3-hydroxy-desthio	Prothioconazole -4-hydroxy-desthio
Specificity	The mass spectra for the hydroxyl derivates of JAU 6476-desthio were already obtained in the course of the development of method 00979 and 00979/M001. Please refer to the Appendix 5 of the report. Blank value < 30 % LOQ)	The mass spectra for the hydroxyl derivates of JAU 6476-desthio were already obtained in the course of the development of method 00979 and 00979/M001. Please refer to the Appendix 5 of the report. Blank value < 30 % LOQ)	The mass spectra for the hydroxyl derivates of JAU 6476-desthio were already obtained in the course of the development of method 00979 and 00979/M001. Please refer to the Appendix 5 of the report. Blank value < 30 % LOQ)
Calibration (type, number of data points)	Calibration data presented Calibration line equation presented in solvent and wheat grain number of data points ≥ 5 always > 0.99	Calibration data presented Calibration line equation presented in solvent and wheat grain number of data points ≥ 5 always > 0.99	Calibration data presented Calibration line equation presented in solvent and wheat grain number of data points ≥ 5 always > 0.99
Calibration range	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)
Assessment of matrix effects is presented	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.
Limit of determination/quantification	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg

Overhead shaker	Prothioconazole-5-hydroxy-desthio	Prothioconazole -6-hydroxy-desthio
Specificity	The mass spectra for the hydroxyl derivatives of JAU 6476-desthio were already obtained in the course of the development of method 00979 and 00979/M001. Please refer to the Appendix 5 of the report. Blank value < 30 % LOQ)	The mass spectra for the hydroxyl derivatives of JAU 6476-desthio were already obtained in the course of the development of method 00979 and 00979/M001. Please refer to the Appendix 5 of the report. Blank value < 30 % LOQ).
Calibration (type, number of data points)	Calibration data presented Calibration line equation presented in solvent and wheat grain number of data points ≥ 5 always > 0.99	Calibration data presented Calibration line equation presented in solvent and wheat grain number of data points ≥ 5 always > 0.99
Calibration range	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)	Excellent linear correlation between the injected amount and detector response was observed within the range of 0.05 to 10 $\mu\text{g/L}$ (corresponding to 0.005 to 1.0 mg/kg)
Assessment of matrix effects is presented	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.	Possible matrix effects were compensated by the use of stable isotope internal standards. Therefore, it was not necessary to prepare the calibration standards in the matrix.
Limit of determination/quantification	LOQ: 0.01 mg/kg	LOQ: 0.01 mg/kg

Conclusion

The method meets all guideline criteria for data generation methods. It allows determination of residues of prothioconazole- α -hydroxy-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-5-hydroxy-desthio and prothioconazole-6-hydroxy-desthio in/on plant matrices with a limit of quantification (LOQ) of 0.01 mg/kg using either an overhead-shaker or an Ultra Turrax for the extraction process.

A 2.1.1.1.6.5 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.6.6 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.6.7 Method validation 3

Comments of zRMS:	The analytical method 01013 was validated for the determination of residues of prothioconazole-desthio in/on plant material (citrus fruit, pea seed, wheat grain, rape seed and corn green material) with a limit of quantification of 0.01 mg/kg. The stability of standards and extracts has not been specified. However, the method is acceptable and considered fit for purpose.
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Reference: KCP 5.1.2/11

Report Analytical method 01013 for the simultaneous determination of residues of the

active Items BYF00587, prothioconazole, tebuconazole, trifloxystrobin and the metabolites BYF00587-desmethyl, JAU6476-desthio (SXX0665) and CGA321113 in/on plant material by HPLC-MS/MS, Brumhard, B.; Stuke, S., 2016, Report No. M-283439-04-1

Guideline(s): SANCO/3029/99
Deviations: Not specified
GLP: Yes
Acceptability: Yes

Materials and methods

All analytes were extracted from plant materials using a mixture of acetonitrile/water (4/1; v/v, containing cysteine hydrochloride) by high-speed blending. After filtration of the extract, the stable isotopically labeled analytes were added. The solution was made up to volume, diluted and subjected to reversed phase HPLC-MS/MS without a further clean-up step. Prothioconazole-desthio was detected using electrospray ionization in the positive ion mode (ESI+). Residues were quantified using internal stable labeled standards. The following transition ions were used for prothioconazole-desthio:

for quantification $m/z = 312 \rightarrow m/z = 70$
for confirmation $m/z = 312 \rightarrow m/z = 125$.

Results and discussions

Table A 57: Recovery results from method validation of Prothioconazole-desthio using the analytical method

Matrix	Analyte	FortificationLevel [mg/kg]	Recoveries [%]						RSD [%]	n
			Individual					Mean		
Prothioconazole- desthio (1 st MRM)	Citrus fruit	0.01	107	105	103	105	98	104	3.3	5
		0.10	100	84	98	96	100	96	7.0	5
	Overall							100	6.6	10
	Pea, green seed	0.01	114	114	97	102	109	107	7.0	5
		0.10	96	99	100	100	102	99	2.2	5
	Overall							103	6.4	10
	Rape seed	0.01	94	101	89	107	95	97	7.1	5
		0.10	88	96	91	101	98	95	5.6	5
	Overall							96	6.2	10
	Wheat grain	0.01	103	97	97	97	99	99	2.6	5
		0.10	100	91	96	94	98	96	3.6	5
	Overall							97	3.4	10
	Corn green material	0.01	116	118	113	119	107	115	4.2	5
		0.10	100	99	97	102	102	100	2.1	5
Overall							107	7.9	10	
Prothioconazole- desthio (2 nd MRM)	Citrus fruit	0.01	103	121	114	105	101	109	7.8	5
		0.10	101	89	98	98	104	98	5.7	5
	Overall							103	8.5	10
	Pea, green seed	0.01	95	95	98	106	105	100	5.4	5
		0.10	107	107	112	106	113	109	3.0	5
	Overall							104	6.1	10
	Rape seed	0.01	97	88	97	97	100	96	4.7	5
		0.10	88	98	90	103	94	95	6.4	5
	Overall							95	5.4	10
	Wheat grain	0.01	96	104	100	94	92	97	5.0	5
		0.10	95	91	90	89	93	92	2.6	5
	Overall							94	4.9	10
	Corn green material	0.01	108	113	109	105	98	107	5.2	5
		0.10	96	95	94	95	99	96	2.0	5
Overall							101	6.8	10	

* Limit of quantitation (LOQ), defined by the lowest validated fortification level

Table A 58: Characteristics for the analytical method used for validation of prothioconazole-desthio

	Prothioconazole-desthio
Specificity	Three untreated control samples of different origins were examined. All residues of the test items were below 30% of the LOQ (0.01 mg/kg).
Calibration (type, number of data points)	The internal standard procedure, using stable isotopically labeled internal standards was used for calibration. The calibration data obtained justified using the single point calibration method for the calculating of the residues. However, an appropriate bracketing standard concentration, corresponding to the order of magnitude of the residues should be used for quantitation and it should be noted that the concentration of the stable isotopically labeled internal standard in the calibration and sample solutions must be kept at a similar level.
Calibration range	The correlation between the injected amount of substance and the detector response was linear for solvent standards ranging from 0.005 to 50 µg/L. The correlation coefficient of the 1/x weighted linear regression was 0.9995. Possible matrix effects were eliminated by the internal standard procedure using isotopically stable labeled standards.
Assessment of matrix effects is presented	Not relevant – Isotopically labelled internal standards used to compensate for matrix effects.

Limit of determination/quantification	The lowest fortification level experimentally tested, at which acceptable recovery data have been obtained in all matrices was 0.01 mg/kg. This level corresponds per definition to the limit of quantitation (LOQ) for the determination of prothioconazole-desthio. The Limit of Detection (LOD) ranged between 0.0010 and 0.0028 mg/kg.
Stability of standards and extracts	Not specified

Conclusion

The method was developed for determination of residues of prothioconazole-desthio in/on plant material (citrus fruit, pea seed, wheat grain, rape seed and corn green material) with a limit of quantification of 0.01 mg/kg in all tested material. This method was successfully validated according to the European requirements (SANCO/3029/99; 96/46/EC of 16 July 1996 amending Directive 91/414/EEC). In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled, with the exception of stability of standards and extracts. Nevertheless, the analytical method has been demonstrated as fit for purpose.

A 2.1.1.1.6.8 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.6.9 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.1.1.7 Azoxystrobin and its metabolite in honey

A 2.1.1.1.7.1 Method validation

Comments of zRMS:	The study of Syngenta method RAM 305/03 as described in Syngenta Report Number: T011298-06-REG for azoxystrobin and its metabolite R230310 in honey (Bocksch S., 2008) has been reviewed at EU level. The analytical method has been validated for beer, wheat flour and various crop matrices and honey with LOQ 0.01 mg/kg. The method is acceptable and considered fit for purpose.
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Reference: KCP 5.1.2/20

Report Azoxystrobin (ICI5504) and Cyproconazole (SAN619) residues in honey following exposure of bees to treated winter oil-seed rape in Germany during 2007, Bocksch S., 2008, Report N°T011298-06-REG

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

Deviations: No

GLP: Yes

Acceptability: Yes

Reference: KCP 5.1.2/22

Report	Determination of Residues of Azoxystrobin and R230310 (z-isomer) in Honey after Two Applications of A12705B to Winter Oilseed rape at 5 Sites in Central and Southern Europe in 2021, Appeltauer A., 2022, Report No. S21-01128
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and method

2g of honey sample were diluted with 20 mL acetonitrile/ultra pure water (90/10, % v/v). The dilution is centrifuged and clean up by C18 SPE cartridge. Quantification of azoxystrobin and R230310 (Z-azoxystrobin) was performed by use of HPLC-MS/MS detection. The limit of quantification (LOQ) was 0.01 mg/kg for both analytes.

Analytical conditions

Chromatographic conditions

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

Column: Kromasil C18, 50 mm x 3.2 mm, 5 µm

Mobile phase: 0.2% v/v acetic acid in water / Acetonitrile (50/50, % v/v)

Flow: 1.0 mL/min

Injection volume: 20 µL

Mass spectrometric conditions

System: Sciex API 4000

Mass transitions

Azoxystrobin: 404.2 → 372.4 (m/z), 404.2 → 343.8 (m/z)

R230310: 404.2 → 372.4 (m/z), 404.2 → 343.8 (m/z)

Results and discussions

Table A 59: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin				
Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404.2 → 372.4 m/z (Primary mass transition)				
0.01	78, 77, 75, 81, 71	76	4.8	5
0.1	83, 82, 83, 89, 82	84	3.4	5
Overall		80	6.3	10
Transition m/z 404.2 → 343.8 m/z (Secondary mass transition)				
0.01	98, 93, 95, 103, 90	96	5.2	5
0.1	83, 80, 81, 90, 85	84	4.5	5
Overall		90	8.4	10

Table A 60: Recovery results from method validation of R230310 using the analytical method

R230310				
Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404.2 → 372.4 m/z (Primary mass transition)				
0.01	75, 83, 80, 84, 73	79	6.2	5
0.1	92, 92, 92, 94, 88	92	2.3	5
Overall		85	9.2	10
Transition m/z 404.2 → 343.8 m/z (Secondary mass transition)				
0.01	79, 82, 74, 94, 85	83	9.3	5
0.1	90, 91, 91, 93, 89	91	1.7	5
Overall		87	7.8	10

Table A 61: Characteristics for the analytical method used for validation of Azoxystrobin and R230310 in honey

	Azoxystrobin	R230310
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 404.2 → 372.4 and m/z 404.2 → 343). The blank value and the control sample value at the expected retention times of prothioconazole did not exceed 30% of the LOQ. Representative chromatograms (calibration solution, control sample, samples fortified at the LOQ and treated residue samples) for the quantification transition.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404.2 → 372.4 and m/z 404.2 → 343). The blank value and the control sample value at the expected retention times of prothioconazole did not exceed 30% of the LOQ. Representative chromatograms (calibration solution, control sample, samples fortified at the LOQ and treated residue samples) for the quantification transition.
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A62 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A62 below.
Calibration range	Linearity was confirmed over the following calibration range : 0.00005 – 0.00150 µg/mL (n = 5), corresponding to fortification levels of 0.005 – 0.15 mg/kg. This covers the range of 50 % of the LOQ and at least + 20 % of the highest analyte concentration. However, 30% of the LOQ can be estimated from the chromatograms and residue levels in the control samples were demonstrated to be below 30% of the LOQ.	Linearity was confirmed over the following calibration range : 0.00005 – 0.00150 µg/mL (n = 6), corresponding to fortification levels of 0.005 – 0.15 mg/kg. This covers the range of 50 % of the LOQ and at least + 20 % of the highest analyte concentration. However, 30% of the LOQ can be estimated from the chromatograms and residue levels in the control samples were demonstrated to be below 30% of the LOQ.
Assessment of matrix effects is presented	Yes (unsignificant)	Yes (unsignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.01mg/kg.	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.01mg/kg.
Stability of standards and extracts	Not assessed within this study. Final extracts were stored at 0 – 9°C for a maximum of 1 day before analysis. The recoveries of the analytes in final sample extracts were within the acceptable range of 70 – 120%, measured against freshly fortified standards, thus proving stability in accordance with SANTE/2020/12830, Rev. 1.	Not assessed within this study. Final extracts were stored at 0 – 9°C for a maximum of 1 day before analysis. The recoveries of the analytes in final sample extracts were within the acceptable range of 70 – 120%, measured against freshly fortified standards, thus proving stability in accordance with SANTE/2020/12830, Rev. 1.

Table A 62: Linearity of detector response

Analyte	Transition	Linearity data
Azoxystrobin	404.2 → 372.4 m/z	$y = 71696411 x + 1130$, $r = 0.9996$ (n = 5)
R230310	404.2 → 372.4 m/z	$y = 61139017 x + 1180$, $r = 0.9981$ (n = 6)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of azoxystrobin and R230310 in honey. The Limit of Quantification was 0.01 mg/kg for both analytes.

A 2.1.1.1.7.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.7.3 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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.

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

A 2.1.1.1.8 Azoxystrobin in oilseed rape pollen and nectar

A 2.1.1.1.8.1 Method validation

Comments of zRMS:	The analytical method was fully and successfully validated in oilseed rape specimens (pollen and nectar) in compliance with SANCO/825/00 rev.8.1 of and SANCO/3029/99 rev.4 (10 spiked samples were performed: 5 at the LOQ level, 5 at ten times the LOQ level; 2 control samples and a reagent blank). LOQ (Limit of quantification) of azoxystrobin: 0.005 mg/kg for each matrix. The recovery mean, and furthermore each individual recovery, at each recovery level (0.005, 0.2 and 2.0 mg/kg) were all between 70% and 110% at each level of fortification and for each matrix. The method is acceptable according to the SANTE/2020/12830 rev.2.
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Reference:	KCP 5.1.2/21
Report	Magnitude of the residue of azoxystrobin in oilseed rape pollen and nectar Raw Agricultural Commodity after two foliar applications of ALB 121 in Southern Europe - 2018, Lebrun F., 2019, Report No. 349-2018
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Sample of nectar and pollen were extracted by agitation in acetonitrile (and ultra-pure water for pollen). Pollen extracts were purified by dispersive solid phase extraction. Quantification of azoxystrobin was performed by use of HPLC-MS/MS detection. The limit of quantification (LOQ) was 0.005 mg/kg for both matrices.

Analytical conditions

Chromatographic conditions

System: LC-MS/MS Shimadzu

Column: Hydro RP, C18, 100 mm x 3.0 mm, 2.5 µm

Mobile phase A: Ultra-pure water/acetic acid (100/0.1, v/v) + 5 mM ammonium acetate

Mobile phase B: Methanol/acetic acid (100/0.1, v/v) + 5 mM ammonium acetate

Flow: 0.7 mL/min

Column temperature: 60°C

Injection volume: 5 µL

Gradient:

Time (min)	% A	% B
0.0	90	10
0.1	90	10

4.0	0	100
6.0	0	100
6.5	90	10
8.0	90	10

Retention time:

Azoxystrobin: About 4.1 – 4.2 min

Mass spectrometric conditions

System: AB Sciex QTRAP 5500

Polarity: Positive ion mode

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

Capillary voltage: 5500V

Ionspray turbo heater (TEM): 550°C

Mass transitions

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 63: Recovery results from method validation of azoxystrobin using the analytical method

Matrix	Azoxystrobin				
	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Pollen	Transition m/z 404 → 372 m/z (Primary mass transition)				
	0.005	113, 85, 76, 89, 69	86	19	5
	0.05	86, 84, 92, 87, 88	87	3	5
	Overall		87	13	-
	Transition m/z 404 → 344 m/z (Secondary mass transition)				
	0.005	103, 81, 68, 85, 63	80	20	5
	0.05	86, 81, 90, 85, 91	87	5	5
	Overall		83	14	-
Nectar	Transition m/z 404 → 372 m/z (Primary mass transition)				
	0.005	85, 86, 83, 83, 82	84	2	5
	0.05	89, 89, 91, 92, 88	90	2	5
	Overall		87	4	-
	Transition m/z 404 → 344 m/z (Secondary mass transition)				
	0.005	83, 85, 84, 83, 87	85	2	5
	0.05	87, 87, 89, 89, 88	88	1	5
	Overall		83	3	-

Table A 64: Characteristics for the analytical method used for validation of Azoxystrobin in nectar and pollen

	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). The specificity was checked by the analysis of untreated sample and reagent blank. Interferences were less than 30% of the LOQ. The solvent blank showed that no interference due to the reagents was detected. Representative chromatograms (lowest calibration level, control sample, samples fortified at the LOQ and treated residue samples) for the quantification transition.
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A65 below.
Calibration range	Linearity was confirmed over the following calibration range 0.03 – 8 µg/L (n = 5) thus covers the range from 30 % of the LOQ to 20 % of the highest level.
Assessment of matrix effects is presented	No
Limit of	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and

	Azoxystrobin
determination/quantification	precision. The LOQ was 0.005mg/kg. The LOD is defined as 30% of the LOQ. The LOD was 0.0015 mg/kg
Stability of standards and extracts	/

Table A 65: Linearity of detector response

Matrix	Transition	Linearity data
Pollen	404 → 372 m/z	$y = 312861 x + 4581.13$, $r = 0.9993$ (n = 8)
	404 → 344 m/z	$y = 84696.88 x + 1452.94$, $r = 0.9996$ (n = 8)
Nectar	404 → 372 m/z	$y = 447621.92 x + 2780.57$, $r = 0.9998$ (n = 8)
	404 → 344 m/z	$y = 122589.00 x + 578.26$, $r = 0.9998$ (n = 8)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of azoxystrobin in pollen and nectar. The Limit of Quantification was 0.005 mg/kg for both matrices.

A 2.1.1.1.8.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.8.3 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.1.9 Description of analytical methods for the determination of residues in honey matrices

A 2.1.1.1.9.1 Method validation

Comments of zRMS:	For prothioconazole only, specimens extraction and determination of residues were performed according to the method that was previously validated in S20-09747. For azoxystrobin and spray solution, specimens extraction and determination of residues were developed and validated in the current analytical phase. The LOQ was 0.01 mg/kg in pollen and nectar for PTZ and azoxystrobin and 67 mg/L in spray solutions. The method is acceptable according to the SANTE/2020/12830 rev.2.
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Reference: KCP 5.1.2/12

Report A semi-field study to evaluate potential effects on the honey bee (*Apis mellifera* L.) after two application of CA3301 and CA3642 in winter oil seed rape in Germany 2022, Bocksch S., 2023, Report N°S21-00461

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and method

Samples of pollen were extracted with acetonitrile/water and cysteine hydrochloride solution. A salt

mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken to obtain phase separation after centrifugation. An aliquot of the acetonitrile phase was cleaned by adding primary secondary amine (PSA) and GCB. After centrifugation the cleaned extract was diluted with methanol/water (40/60 v/v) containing 50 g/L cysteine hydrochloride solution. Quantification of azoxystrobin and prothioconazole* was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) was 0.01mg/kg for both analytes.

Samples of nectar were dissolved in acetonitrile/water and cysteine hydrochloride solution and diluted in methanol/water (40/60 v/v). Quantification of azoxystrobin and prothioconazole* was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) was 0.01mg/kg for both analytes.

*Prothioconazole extraction and determination in pollen and nectar was validated in study S20-09747.

Samples of spray solutions were diluted with acetonitrile/water (1:1, v/v) + 10% of 250 g/L L-cysteine solution and methanol/water (40/60 v/v). Quantification of azoxystrobin and prothioconazole was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) was 67 mg/L for both analytes.

Analytical conditions

Chromatographic conditions

System: 1290 Infinity II Binary LC System, Agilent technologies

Pre-column: Phenomenex UHPLC guard column with 4mm C18 cartridge

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

Mobile phase A: 0.1% v/v formic acid in water

Mobile phase B: 0.1% v/v acid formic in acetonitrile

Flow: 0.8 mL/min

Column temperature: 40°C

Injection volume: 10 µL

Gradient:

Time (min)	% A	% B
0	80	20
2.0	80	20
5.0	10	90
6.5	10	90
7.5	80	20
8.5	80	20

Divert valve: 0.0 min to 3.5 min to waste, 3.5 min to 5.0 min to MS, 5.0 min to 8.5 min to waste

Retention time:

Azoxystrobin: About 4.1 min

Prothioconazole: About 4.3 min

Mass spectrometric conditions

System: Sciex API 6500+ Linear Ion Trap Quadrupole

Ionisation type: Electrospray ionisation (ESI, TurboIonSpray)

Polarity: Positive ion mode

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

Capillary voltage: 5000V

Ionspray turbo heater (TEM): 550°C

Mass transitions

Prothioconazole: 344 → 154 (m/z) for quantification, 346 → 227 (m/z) for confirmation

Azoxystrobin: 404 → 344 (m/z) for quantification, 404 → 372 (m/z) for confirmation

Prothioconazole-desthio*: 312 → 125

* Since azoxystrobin has the same retention time as Prothioconazole-desthio (see study report S20-09747), the prothioconazole-desthio transition was included when injecting just an azoxystrobin standard just to confirm that there is no presence of one analyte in the other

Results and discussions

Table A 66: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole							
Matrix	Fortification Level (mg/kg or ng/mL*)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 344 → 154 m/z (Proposed for Quantification)							
Nectar	0.01	100, 97, 89, 102, 96	97	5	5	99	4
	0.1	101, 103, 101, 98, 99	100	2	5		
	1	99, 101, 94, 104, 104	100	4	5		
Pollen	0.01	85, 92, 89, 92, 100	92	6	5	92	9
	1	78, 93, 96, 100, 89	91	9	5		
	100	112, 84, 88, 94, 86	93	12	5		
Spray solutions	66666	101, 103, 98, 100, 96	100	3	5	96	5
	907715	90**, 93**, 90**, 90, 96	92	3	5		

*For spray solutions

** Mean of two injections

Table A 67: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg or ng/mL*)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 344 m/z (Proposed for Quantification)							
Nectar	0.01	111, 105, 107, 108, 110	108	2	5	107	2
	0.1	102, 107, 106, 107, 106	106	2	5		
	1	109, 107, 101, 108, 108	107	3	5		
Pollen	0.01	82, 85, 86, 92, 97	88	7	5	94	8
	1	86, 96, 98, 101, 93	95	6	5		
	100	104, 93, 88, 105, 99	98	7	5		
Spray solutions	66666	98, 103, 107, 100, 104	102	3	5	102	3
	908282	100**, 100**, 101**, 104, 104	102	2	5		

*For spray solutions

** Mean of two injections

Table A 68: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in pollen, nectar and spray solutions

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 154 for quantification and m/z 346 → 227 for confirmation).	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 344 for quantification and m/z 404 → 372 for confirmation). No interference at the retention time of azoxystrobin above 30% of the LOQ were observed in the reagent blank and control sample. Representative chromatograms (lowest calibration level, control sample, samples fortified at the LOQ and samples fortified at 10x LOQ) and product ion spectra are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards for pollen and nectar and solvent calibration standard for spray solutions. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.995. Please see table A 69 below.	The linearity of the method was demonstrated using matrix-matched calibration standards for pollen and nectar and solvent calibration standard for spray solutions. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.995. Please see table A 69 below.

	Prothioconazole	Azoxystrobin
Calibration range	Linearity was confirmed over the following calibration range : - Pollen: 0.1 – 9.0 ng/mL (n = 9), corresponding to 0.003 – 0.27 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration - Nectar: 0.03 – 3.0 ng/mL (n = 8), corresponding to 0.003 – 0.3 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration - Spray sol.: 0.04 – 3.0 ng/mL (n = 8), corresponding to 20 – 1500 mg/L thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration	Linearity was confirmed over the following calibration range : - Pollen: 0.1 – 9.0 ng/mL (n = 9), corresponding to 0.003 – 0.27 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration - Nectar: 0.03 – 3.0 ng/mL (n = 8), corresponding to 0.003 – 0.3 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration - Spray sol.: 0.04 – 3.0 ng/mL (n = 8), corresponding to 20 – 1500 mg/L thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration
Assessment of matrix effects is presented	Yes (significant only for pollen)	Yes (unsignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.01 mg/kg in pollen and nectar and 67 mg/L in spray solutions. The LOD was defined as the lowest calibration level. The LOD is 0.003 mg/kg (30% of the LOQ) in pollen and nectar.	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.01 mg/kg in pollen and nectar and 67 mg/L in spray solutions. The LOD was defined as the lowest calibration level. The LOD is 0.003 mg/kg (30% of the LOQ) in pollen and nectar.
Stability of standards and extracts	<u>Extracts:</u> Nectar extracts are considered to be stable when stored at 1°C to 10°C for 6 days. Spray solutions dilutions are considered to be stable when stored at 1°C to 10°C for 8 days. The pollen samples were injected within 24 hours after extraction. No storage stability is required. <u>Standards:</u> The solvent solutions prepared in acetonitrile were considered to be stable when stored at 1°C to 10°C in the dark for 80 days.	<u>Extracts:</u> Nectar extracts are considered to be stable when stored at 1°C to 10°C for 6 days. Spray solutions dilutions are considered to be stable when stored at 1°C to 10°C for 8 days. The pollen samples were injected within 24 hours after extraction. No storage stability is required. <u>Standards:</u> The solvent solutions prepared in acetonitrile were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.

Table A 69: Linearity of detector response

Matrix	Analyte	Transition	Linearity data
Nectar	Prothioconazole	344 → 154 m/z (Quantification)	$y = 1.41.10^5 x - 614, r = 0.9997 (n = 8)$
	Azoxystrobin	404 → 344 m/z (Quantification)	$y = 7.43.10^5 x + 3.1.10^3 r = 0.9996 (n = 8)$
Pollen	Prothioconazole	344 → 154 m/z (Quantification)	$y = 1.06.10^5 x + 2.10^3, r = 0.9973 (n = 9)$
	Azoxystrobin	404 → 344 m/z (Quantification)	$y = 8.46.10^5 x + 4.57.10^3 r = 0.9999 (n = 9)$
Spray solutions	Prothioconazole	344 → 154 m/z (Quantification)	$y = 1.5.10^5 x + 1.18.10^3, r = 0.9997 (n = 8)$
	Azoxystrobin	404 → 344 m/z (Quantification)	$y = 8.96.10^5 x + 6.45.10^3 r = 0.9998 (n = 8)$

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in pollen, nectar and spray solutions. The Limit of Quantification in pollen and nectar was 0.01mg/kg for both analytes. The Limit of Quantification in spray solution was 67 mg/L for both analytes.

A 2.1.1.1.9.2

Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.1.9.3 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2 Description of analytical methods in support of ecotoxicologic test

A 2.1.1.2.1 Method validation 1: Sucrose solution

Comments of zRMS:	<p>A method was successfully validated for determination of CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC) in feeding solutions. Quantification was performed by LC-MS/MS.</p> <p>The limit of quantification (LOQ) of the analytical method was 15.0 mg test item/kg in 50% w/v aqueous sucrose solution (corresponding to 2.08 mg prothioconazole/kg and 2.11 mg azoxystrobin/kg) with a limit of detection (LOD) set at 0.525 mg/kg for both analytes.</p> <p>The method is fit for purpose.</p>
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Reference:	KCP 5.1.2/13
Report	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Honey Bee (<i>Apis mellifera</i> L.) chronic oral toxicity test (10-Day feeding) under laboratory conditions, Gimeno I., 2022, Report N°S21-04081
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and method

Samples of 50% (w/v) aqueous sucrose solution were diluted with acetonitrile/water and if necessary with matrix blank extract to be within the calibration range. Quantification of azoxystrobin and prothioconazole was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) was 15 mg test item/kg corresponding to 2.11 mg azoxystrobin/kg and 2.08 mg prothioconazole/kg.

Analytical conditions

Chromatographic conditions

System: Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP
Pre-column: Phenomenex, UHPLC guard column with 2.1mm C18 cartridge
Column: Supelco Ascentis Express C18, 50 mm x 2.1 mm, 2.7 µm
Mobile phase A: 0.1% v/v formic acid in water
Mobile phase B: Methanol
Flow: 0.5 mL/min
Column temperature: 40°C
Injection volume: 5 µL
Gradient:

Time (min)	% A	% B
0.01	95	5
0.50	95	5
3.00	5	95
4.00	5	95
4.10	95	5
5.00	95	5

Divert valve: 0.0 min to 2.4 min to waste, 2.4 min to 3.6 min to MS, 3.6 min to 5.0 min to waste
Retention time:

Azoxystrobin: About 2.9 min
Prothioconazole: About 3.2 min

Mass spectrometric conditions

System: Sciex API 5500

Polarity: Positive ion mode

Scan type: Multiple Reaction Monitoring (MRM)

Capillary voltage: 5500V

Ionspray turbo heater (TEM): 550°C

Mass transitions

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation

Results and discussions

Table A 70: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole					
Matrix	Fortification Level (mg test item/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 344 → 189 m/z (Proposed for Quantification)					
50 % (w/v) aqueous sucrose solution	15.0	86, 90, 86, 90, 85	87	3	5
	7194	97, 95, 91, 93, 94	94	2	5

Table A 71: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin					
Matrix	Fortification Level (mg test item/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404 → 372 m/z (Proposed for Quantification)					
50 % (w/v) aqueous sucrose solution	15.0	90, 92, 91, 89, 87	90	2	5
	7194	96, 96, 96, 97, 98	97	1	5

Table A 72: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in sucrose solutions

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). The blank values and the reagent blank value at the expected retention times of prothioconazole of the control sample did not exceed the LOD. Since blank peaks were not observed, blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample, samples fortified at the LOQ and treated residue samples) for the quantification transition and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). The blank values and the reagent blank value at the expected retention times of azoxystrobin of the control sample did not exceed the LOD. Since blank peaks were not observed, blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample, samples fortified at the LOQ and treated residue samples) for the quantification transition 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 performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A73 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A73 below.
Calibration range	Linearity was confirmed over the following	Linearity was confirmed over the following

	Prothioconazole	Azoxystrobin
	calibration range : 0.5 – 6.5 ng/mL (n = 7), corresponding to fortification levels of 0.525 – 6.83 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration.	calibration range : 0.5 – 6.5 ng/mL (n = 7), corresponding to fortification levels of 0.525 – 6.83 mg/kg thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration.
Assessment of matrix effects is presented	Yes (unsignificant)	Yes (unsignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 15.0 mg test item/kg corresponding to 2.08 mg prothioconazole/kg. The LOD was defined as the lowest calibration level. The LOD is 0.525 mg/kg.	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 15.0 mg test item/kg corresponding to 2.11 mg prothioconazole azoxystrobin/kg. The LOD was defined as the lowest calibration level. The LOD is 0.525 mg/kg.
Stability of standards and extracts	<u>Extracts:</u> Sample extracts (at LOQ level and high level) are considered to be stable when stored at 1°C to 10°C for 4 days. <u>Standards:</u> The working solutions prepared in acetonitrile (i.e stock, fortification and calibration solutions) were considered to be stable when stored at 1°C to 10°C in the dark for 31 days.	<u>Extracts:</u> The interval from preparation of the final extracts to injection did not exceed 24 hours. Due to the shortness of the interval any effect on the results due to a possible instability of the analyte in final sample extracts are considered to be insignificant. <u>Standards:</u> The working solutions prepared in acetonitrile (i.e stock, fortification and calibration solutions) were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.

Table A 73: Linearity of detector response

Analyte	Transition	Linearity data
Prothioconazole	344 → 189 m/z (Quantification)	$y = 2.33.10^4 x - 841$, $r = 0.9995$ (n = 7)
Azoxystrobin	404 → 372 m/z (Quantification)	$y = 2.84.10^5 x + 1.07.10^4$ $r = 0.9998$ (n = 7)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in 50% (w/v) aqueous sucrose solution. The Limit of Quantification was 15.0 mg test item/kg corresponding to 2.11 mg azoxystrobin/kg and 2.08 mg prothioconazole/kg. The LOD was set at 0.525 mg/kg for both analytes.

A 2.1.1.2.1.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.1.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.2 Method validation 2: Deionised water

Comments of zRMS:	A method was successfully validated for determination of CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC) in deionised water.
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	The limit of quantification (LOQ) of the analytical method was 90.0 mg test item/L (corresponding to 12.5 mg prothioconazole/L and 12.7 mg azoxystrobin/L) with a limit of detection (LOD) set at 3.50 mg/L for both analytes. The method is fit for purpose.
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Reference:	KCP 5.1.2/14
Report	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Honey Bee (<i>Apis mellifera</i> L.) larval toxicity test following repeated exposure under laboratory conditions, Gimeno I., 2022, Report N°S21-04082
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and method

Samples of deionised water were extracted then diluted with acetonitrile/water (1:1 v/v) mixture and if necessary with matrix blank extract to be within the calibration range. Quantification was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) was 90.0 mg test item/L, corresponding to 12.7 mg azoxystrobin/L and 12.5 mg prothioconazole/L.

Analytical conditions

Chromatographic conditions

System: Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP
Pre-column: Phenomenex UHPLC guard column with 2.1 mm C18 cartridge
Column: Supelco Ascentis Express C18, 50 mm x 2.1 mm, 2.7 µm
Mobile phase A: 0.1% v/v formic acid in water
Mobile phase B: Methanol
Flow: 0.5 mL/min
Column temperature: 40°C
Injection volume: 5 µL

Time (min)	% A	% B
0	95	5
0.5	95	5
3.0	5	95
4.0	5	95
4.1	95	5
5.0	95	5

Divert valve: 0.0min to 2.4 min to waste, 2.4min to 3.6min to MS, 3.6min to 5.0 min to waste

Retention time:

Azoxystrobin: About 2.9 min

Prothioconazole: About 3.2 min

Mass spectrometric conditions

System: Sciex API 5500
Polarity: Positive ion mode
Scan type: Multiple Reaction Monitoring (MRM)
Capillary voltage: 5500V
Ionspray turbo heater (TEM): 550°C
Mass transitions

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation

Results and discussions

Table A 74: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole				
Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 344 → 189 m/z (Proposed for Quantification)				
90.0	96, 94, 97, 95, 90	94	3	5
47000	96, 94, 93, 96, 97	95	2	5

A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.

Table A 75: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin				
Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404 → 372 m/z (Proposed for Quantification)				
90.0	96, 100, 102, 95, 92	97	4	5
47000	92, 92, 94, 94, 98	94	3	5

A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.

Table A 76: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in deionised water

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the reagent blank sample. The blank values at the expected retention times of prothioconazole did not exceed the LOD. Blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). No interference at the retention time of Azoxystrobin above 30% of the LOQ were observed in the reagent blank sample. The blank values at the expected retention times of azoxystrobin did not exceed the LOD. Blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition 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 performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A77 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A77 below.
Calibration range	Linearity was confirmed over the calibration range 0.7 – 6.5 ng/mL ($n = 7$), covering test samples with a test item concentration of 3.5 to 32.5 mg/L and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in the diluted sample.	Linearity was confirmed over the calibration range 0.7 – 6.5 ng/mL ($n = 7$), covering test samples with a test item concentration of 3.5 to 32.5 mg/L and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in the diluted sample.
Assessment of matrix effects is presented	Yes (insignificant)	Yes (insignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 90.0 mg test item/L,	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 90.0 mg test item/L,

	Prothioconazole	Azoxystrobin
	corresponding to 12.5 mg prothioconazole/L. The LOD was defined as the lowest calibration level. The LOD is 3.50 mg prothioconazole/L (30% of the LOQ).	corresponding to 12.7 mg azoxystrobin/L. The LOD was defined as the lowest calibration level. The LOD is 3.50 mg azoxystrobin/L (30% of the LOQ).
Stability of standards and extracts	Extracts (analytes in the final dilution) were considered to be stable when stored at 1°C to 10°C for 3 days in the dark. Working solutions (i.e stock, fortification and solvent calibration solutions) prepared in acetonitrile were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.	Extracts (analytes in the final dilution) were considered to be stable when stored at 1°C to 10°C for 3 days in the dark. Working solutions (i.e stock, fortification and solvent calibration solutions) prepared in acetonitrile were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.

Table A 77: Linearity of detector response

Analyte	Transition	Linearity data
Prothioconazole	344 → 189 m/z (Quantification)	$y = 3.35.10^4 x - 882$, $r = 0.9997$ (n = 7)
Azoxystrobin	404 → 372 m/z (Quantification)	$y = 3.2.10^5 x + 8.28.10^3$ $r = 0.9991$ (n = 7)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in deionised water. The Limit of Quantification was 90.0 mg test item/L for all analytes (corresponding to 12.7 mg Azoxystrobin/L and 12.5 mg Prothioconazole/L).

A 2.1.1.2.2.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.2.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.3 Method validation 3: Sucrose solution

Comments of zRMS:	<p>A method was successfully validated for determination of CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC) in 50% (w/v) in aqueous sucrose solutions and in 0.1% Triton X solution.</p> <p>The LOQ was 200.0 mg test item/L (corresponding to 28.1 mg azoxystrobin/L and 27.7 mg prothioconazole/L) in 50% aqueous sucrose dilution and 2000 mg test item/L (corresponding to 281 mg azoxystrobin/L and 277 mg prothioconazole/L) in 0.1% Triton X solution.</p> <p>The method is fit for purpose.</p>
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Reference:	KCP 5.1.2/15
Report	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Acute oral and contact Toxicity to the Bumblebee <i>Bombus terrestris</i> L., under laboratory conditions, Gimeno I., 2022, Report N°S21-04083
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes

Acceptability: Yes

Materials and methods

Samples of 50% (w/v) aqueous sucrose solutions and samples of 0.1% Triton X solution were diluted with acetonitrile/water (1:1 v/v) mixture and if necessary with matrix blank extract to be within the calibration range. Quantification was performed by use of LC-MS/MS detection.

The limit of quantification (LOQ) of the analytical method for 50% (w/v) aqueous sucrose solution was 200.0 mg test item/L, corresponding to 28.1 mg azoxystrobin/L and 27.7 mg prothioconazole/L.

The limit of quantification (LOQ) of the analytical method for 0.1% Triton X solution was 2000 mg test item/L, corresponding to 281 mg azoxystrobin/L and 277 mg prothioconazole/L.

Analytical conditions

Chromatographic conditions

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

Pre-column: Phenomenex UHPLC guard column with 2.1mm C18 cartridge

Column: Agilent ZORBAX Eclipse XDB-C18, 600bar, 50 mm x 4.6 mm, 1.8 µm

Flow: 0.4 mL/min

Column temperature: 40°C

Injection volume: 3µL respectively 5µL (the injection volume was lowered during the study due to the high intensity of the azoxystrobin signal, injection volume was consistent with each sequence)

Mobile phase A: 0.1% v/v acetic acid in water

Mobile phase B: Methanol

Time (min)	% A	% B
0	80	20
0.3	80	20
2.0	10	90
4.5 0	10	90
4.51	80	20
6.50	80	20

Divert valve: 0.0min to 2.5 min to waste, 2.5min to 4.8min to MS, 4.8min to 6.5 min to waste

Retention time:

Azoxystrobin: About 3.6 min

Prothioconazole: About 4.3 min

Mass spectrometric conditions

System: Sciex API 5500

Polarity: Positive ion mode

Scan type: Multiple Reaction Monitoring (MRM)

Capillary voltage: 4500V

Ionspray turbo heater (TEM): 450°C

Mass transitions

Azoxystrobin: 404 → 344 (m/z) for quantification, 404 → 372 (m/z) for confirmation

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation

Results and discussions

Table A 78: Recovery results from method validation of Azoxystrobin using the analytical method

Azoxystrobin					
Matrix	Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404 → 344 m/z (Proposed for Quantification)					
50% (w/v) aqueous sucrose solution	200 (LOQ)	102, 99, 99, 95, 98	99	3	5
	46000	99, 96, 98, 95, 95	97	2	5
0.1% Triton X Solution	2000 (LOQ)	100, 95, 96, 94, 102	97	4	5
	477000	101, 105, 105, 111, 105	105	3	5

A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.

Table A 79: Recovery results from method validation of prothioconazole using the analytical method

Matrix	Prothioconazole				
	Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 344 → 189 m/z (Proposed for Quantification)					
50% (w/v) aqueous sucrose solution	200 (LOQ)	90, 86, 93, 84, 92	89	4	5
	46000	96, 101, 108, 105, 103	103	4	5
0.1% Triton X Solution	2000 (LOQ)	93, 95, 99, 95, 100	96	3	5
	477000	100, 100, 106, 99, 101	101	3	5

A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.

Table A 80: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in 50% aqueous sucrose solution and 0.1% Triton X

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the reagent blank sample. The blank values at the expected retention times of prothioconazole did not exceed the LOD. Blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). No interference at the retention time of Azoxystrobin above 30% of the LOQ were observed in the reagent blank sample. The blank values at the expected retention times of azoxystrobin did not exceed the LOD. Blank correction was not necessary. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition and product ion spectra are provided.
Calibration (type, number of data points)	<u>50% aqueous sucrose solution:</u> The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. <u>0.1% Triton X solution:</u> The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A781 below.	<u>50% aqueous sucrose solution:</u> The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. <u>0.1% Triton X solution:</u> The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A81 below.
Calibration range	<u>50% aqueous sucrose solution:</u> Linearity was confirmed over the calibration range 8.1 – 70 ng/mL (n = 7), corresponding to fortification levels of 8 at 70 mg/L and covering the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample. A second linearity was confirmed over the calibration 4 ng/mL to 26 ng/mL (n = 6), corresponding to fortification levels of 8 at 52 mg/L and covering the range from no more than	<u>50% aqueous sucrose solution:</u> Linearity was confirmed over the calibration range 8.1 – 70 ng/mL (n = 7), corresponding to fortification levels of 8 at 70 mg/L and covering the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample. A second linearity was confirmed over the calibration 4 ng/mL to 26 ng/mL (n = 6), corresponding to fortification levels of 8 at 52 mg/L and covering the range from no more than

	Prothioconazole	Azoxystrobin
	<p>30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample.</p> <p><u>0.1% Triton X solution:</u> Linearity was confirmed over the calibration range 8.1 – 70 ng/mL (n = 7), corresponding to fortification levels of 81 at 700 mg/L and covering the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in the diluted sample.</p>	<p>30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample.</p> <p><u>0.1% Triton X solution:</u> Linearity was confirmed over the calibration range 4 – 30 ng/mL (n = 7), corresponding to fortification levels of 80 at 600 mg/L and covering the range from no more than 30 % of the LOQ and at least + 20% of the highest analyte concentration detected in the diluted sample.</p>
Assessment of matrix effects is presented	<p>Yes for 50% (w/v) aqueous solution (significant: matrix enhancement) No for 0.1 % Triton X solution (samples were diluted by at least 10000 with solvent. Matrix effects were considered to be neglectable)</p>	<p>Yes for 50% (w/v) aqueous solution (insignificant) No for 0.1% Triton X solution (samples were diluted by at least 20000 with solvent. Matrix effects were considered to be neglectable)</p>
Limit of determination/quantification	<p><u>50% aqueous sucrose solution:</u> The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 200.0 mg test item/L, corresponding to 27.7 mg prothioconazole/L. The LOD was defined as the lowest calibration level. The LOD is 8.0 mg prothioconazole/L (30% of the LOQ).</p> <p><u>0.1% Triton X solution:</u> The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 2000 mg test item/L, corresponding to 277 mg prothioconazole/L. The LOD was defined as the lowest calibration level. The LOD is 81 mg prothioconazole/L (≤ 30% of the LOQ).</p>	<p><u>50% aqueous sucrose solution:</u> The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 200.0 mg test item/L, corresponding to 28.1 mg azoxystrobin/L. The LOD was defined as the lowest calibration level. The LOD is 8.0 mg azoxystrobin/L (30% of the LOQ).</p> <p><u>0.1% Triton X solution:</u> The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 2000 mg test item/L, corresponding to 281 mg azoxystrobin/L. The LOD was defined as the lowest calibration level. The LOD is 8 mg azoxystrobin/L (≤ 30% of the LOQ).</p>
Stability of standards and extracts	<p>Working solutions (i.e stock, fortification and solvent calibration solutions) prepared in acetonitrile, were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.</p> <p><u>50% aqueous sucrose solution:</u> The interval from preparation of the final extracts to injection did not exceed 24 hours. Due to the shortness of the interval any effect on the results due to a possible instability of the analyte in final sample extracts are considered to be insignificant.</p> <p><u>0.1% Triton X solution:</u> The stability of the analytes in the final extracts was proven by the corresponding concurrent recovery samples, which were stored under the same conditions together with the extracts of the samples for residue analysis and analysed against a freshly prepared calibration curve. The mean recovery values were in the range of 70% – 110%.</p>	<p>Working solutions (i.e stock, fortification and solvent calibration solutions) prepared in acetonitrile were considered to be stable when stored at 1°C to 10°C in the dark for 65 days.</p> <p><u>50% aqueous sucrose solution:</u> Extracts are considered to be stable when stored at 1°C to 10°C for 4 days in the dark.</p> <p><u>0.1% Triton X solution:</u> The stability of the analytes in the final extracts was proven by the corresponding concurrent recovery samples, which were stored under the same conditions together with the extracts of the samples for residue analysis and analysed against a freshly prepared calibration curve. The mean recovery values were in the range of 70% – 110%.</p>

Table A 81: Linearity of detector response

Matrix	Analyte	Transition	Linearity data
50% aqueous sucrose solution	Azoxystrobin	404 → 344 m/z (Quantification)	$y = 6.23 \cdot 10^5 x + 1.68 \cdot 10^6$, $r = 0.9967$ (n = 7) Range : From 8.1 ng/mL to 70 ng/mL $y = 3.7 \cdot 10^5 x + 2.62 \cdot 10^5$, $r = 0.9954$ (n = 6) Range : From 4 ng/mL to 26 ng/mL
	Prothioconazole	344 → 189 m/z (Quantification)	$y = 7.82 \cdot 10^4 x - 2.08 \cdot 10^5$, $r = 0.9957$ (n = 7) Range : From 8.1 ng/mL to 70 ng/mL $y = 3.07 \cdot 10^4 x + 6.97 \cdot 10^3$, $r = 0.9980$ (n = 6) Range : From 4 ng/mL to 26 ng/mL
Acetonitrile/water (1 :1, v/v)	Azoxystrobin	404 → 344 m/z (Quantification)	$y = 5.89 \cdot 10^5 x + 3.45 \cdot 10^5$, $r = 0.9979$ (n = 7)
	Prothioconazole	344 → 189 m/z (Quantification)	$y = 9.65 \cdot 10^4 x - 2.31 \cdot 10^5$, $r = 0.9989$ (n = 7)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in 50% aqueous sucrose solution and 0.1% Triton X solution. The Limit of Quantification was 200.0 mg test item/L (corresponding to 28.1 mg azoxystrobin/L and 27.7 mg prothioconazole/L) in 50% aqueous sucrose dilution and 2000 mg test item/L (corresponding to 281 mg azoxystrobin/L and 277 mg prothioconazole/L) in 0.1% Triton X solution.

A 2.1.1.2.3.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.3.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.4 Method validation 4: Spray solution

Comments of zRMS:	A method was successfully validated for determination of azoxystrobin and prothioconazole in tap water. The Limit of Quantification was 1560 mg test item/L in tap water (219 mg/L for azoxystrobin and 216 mg/L for prothioconazole). The method is fit for purpose.
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Reference: KCP 5.1.2/16

Report CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC): Effects on the Seedling Emergence and Growth of Ten Non-Target Terrestrial Plant Species under Greenhouse Conditions, Huerta F., 2023, Report N°S21-04084

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Samples of spray solution of CA3642 were diluted with acetonitrile and diluted further with acetonitrile/water (1/1 v/v). Quantification was performed by use of LC-MS/MS detection. The limit of quantification (LOQ) of the analytical method was 1560 mg test item/L in tap water, corresponding to 219 mg/L for azoxystrobin and 216 mg/L for prothioconazole in tap water.

Analytical conditions

Chromatographic conditions

System: Agilent Technologies 1100 Infinity

Pre-column: Phenomenex HPLC guard column with 4mm C18 cartridge

Column: Kinetex® C18, 50 mm x 4.6 mm, 2.6 µm

Flow: 0.5 mL/min

Column temperature: 30°C

Injection volume: 10µL

Mobile phase A: Acetonitrile

Mobile phase B: Water containing 0.5% v/v formic acid

Time (min)	% A	% B
0	50	50
0.5	50	50
1.5	80	20
3.5	80	20
4.0	50	50
5.0	50	50

Divert valve: 0.1min to 4.9 min to MS

Retention time:

Azoxystrobin: About 1.5 min

Prothioconazole: About 2.3 min

Mass spectrometric conditions

System: Sciex API 4000

Ionisation type: Electrospray ionization (ESI)

Polarity: Positive mode

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

Capillary voltage: 4500V

Ionspray turbo heater (TEM): 400°C

Mass transitions

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 329 (m/z) for confirmation

Prothioconazole: 344 → 326 (m/z) for quantification, 344 → 189 (m/z) for confirmation

Results and discussions

Table A 82: Recovery results from method validation of Azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall mean recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Tap water	219 (LOQ)	69, 77, 97, 97, 106	89	17	5	96	13
	2863 (mean)	102, 104, 103, 103, 105	103	1	5		

Recoveries are without any blank correction

Table A 83: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole							
Matrix	Fortification Level (mg test item/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall mean recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 344 → 326 m/z (Proposed for Quantification)							
Tap water	219 (LOQ)	92, 102, 100, 98, 102	99	4	5	100	3
	28616 (mean)	101, 102, 99, 100, 104	101	2	5		

Recoveries are without any blank correction

Table A 84: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in tap water

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring one mass transitions (m/z 344 → 326). The blank value at the expected retention time of Prothioconazole did not exceed LOD. Blank correction was not necessary. Reagent blank value did not exceed LOD. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition and product ion spectra are provided.	MS/MS determination was conducted by monitoring one mass transitions (m/z 404 → 372). The blank value at the expected retention time of Azoxystrobin did not exceed LOD. Blank correction was not necessary. Reagent blank value did not exceed LOD. Representative chromatograms (lowest calibration level, control sample and samples fortified at the LOQ) for the quantification transition 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 performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A85 below.	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x-weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A85 below.
Calibration range	Linearity was confirmed over the calibration range 10 – 100 ng/mL (n = 6), corresponding to fortification levels of 40 to 400 mg/L covering the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample.	Linearity was confirmed over the calibration range 10 – 100 ng/mL (n = 7), corresponding to fortification levels of 40 to 400 mg/L covering the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in the diluted sample.
Assessment of matrix effects is presented	Yes (insignificant)	Yes (insignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 216 mg/L for prothioconazole. The LOD was defined as the lowest calibration level. The LOD is 40 mg/L prothioconazole ($\leq 30\%$ of the LOQ).	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 219 mg/L for azoxystrobin. The LOD was defined as the lowest calibration level. The LOD is 40 mg/L prothioconazole azoxystrobin ($\leq 30\%$ of the LOQ).
Stability of standards and extracts	Working solutions (i.e stock, fortification, mix solution) prepared in acetonitrile are considered to be stable when stored at 1°C to 10°C for 65 days Extracts are considered to be stable when stored at 1°C to 10°C for 27 days in the dark.	Working solutions (i.e stock, fortification, mix solution) prepared in acetonitrile are considered to be stable when stored at 1°C to 10°C for 65 days Extracts are considered to be stable when stored at 1°C to 10°C for 27 days in the dark.

Table A 85: Linearity of detector response

Matrix	Analyte	Transition	Linearity data
Acetonitrile/water (1/1, v/v)	Azoxystrobin	404 → 372 m/z (Quantification)	$y = 2.08 \cdot 10^5 x + 2.16 \cdot 10^5$ r = 0.9996 (n = 6)
	Prothioconazole	344 → 326 m/z (Quantification)	$y = 2.06 \cdot 10^4 x - 7.73 \cdot 10^4$, r = 0.9994 (n = 6)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in tap water. The Limit of Quantification was 219 mg/L for azoxystrobin and 216 mg/L for prothioconazole.

A 2.1.1.2.4.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.4.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.5 Analytical method 5: Test medium

Comments of zRMS:	A method was successfully validated for determination of azoxystrobin and prothioconazole in test water samples. The LOQ was 0.00982 mg test item/L, corresponding to 1.38 µg azoxystrobin/L and 1.36 µg prothioconazole/L. The method is fit for purpose.
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Reference:	KCP 5.1.2/17
Report	CA3642 (Prothioconazole 150 g/L + Azoxystrobin 150 g/L SC) – Acute toxicity to rainbow trout (<i>Oncorhynchus mykiss</i>), in a static 96-hour test, [REDACTED], 2023, Report N°20210195
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	Yes: Linearity range doesn't cover the range from 30% to 20% above the highest spike level but covers the range from 30% to 20% above the highest test concentration. The minor deviation to the guideline doesn't impact the study.
GLP:	Yes
Acceptability:	Yes

Materials and methods

The test item concentration in the samples was determined by high pressure liquid chromatography (HPLC) with MS/MS detection using external calibration.

Immediately after sampling, acetonitrile was added to each sample to stabilize the latter during the storage period in frozen conditions. If necessary, the samples were further diluted into the calibration range with test water/acetonitrile (1/1, v/v) mixture. The complete test sample work-up process took place under red light. An aliquot of the diluted samples was analyzed by HPLC with MS/MS detection. The limit of quantification was 0.00982 mg test item/L corresponding to 1.38 µg azoxystrobin/L and 1.36 µg prothioconazole/L.

Analytical conditions

Chromatographic conditions

System: 1290 Infinity II High speed pump, Agilent technologies

Autosampler: Agilent 1290 Infinity II Multisampler

Pre-column: Phenomenex SecurityGuard ULTRA C18

Column: Restek Pinnacle DB AQ C18 AQ C18, 50 mm x 2.1 mm, 1.9 µm

Mobile phase A: 0.1% v/v formic acid in water

Mobile phase B: 0.1% v/v formic acid in acetonitrile

Flow: 0.6 mL/min

Column temperature: 35°C

Injection volume: 20 µL

Injection temperature: 10°C

Time (min)	% A	% B
0	95	5
0.5	70	30
3.0	10	90
4.0	10	90
4.1	95	5

5.0	95	5
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Retention time:

Prothioconazole: About 2.4 min

Azoxystrobin: About 2.3 min

Mass spectrometric conditions

System: AB Sciex / QTRAP 6500

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Capillary voltage: 5000V

Source temperature: 500°C

Mass transitions

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 86: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 344 → 189 <i>m/z</i> (Proposed for Quantification)						
0.00982	108, 112, 113, 115, 113	112	2	5	115	3
3.07	115, 118, 117, 118, 120	118	2	5		
Transition <i>m/z</i> 344 → 154 <i>m/z</i> (Proposed for Quantification)						
0.00982	107, 112, 112, 115, 113	112	2	5	115	4
3.07	117, 118, 119, 119, 120	119	1	5		

Table A 87: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)						
0.00982	114, 113, 115, 115, 119	115	2	5	113	3
3.07	109, 109, 110, 113, 113	111	2	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Quantification)						
0.00982	117, 116, 118, 121, 120	118	2	5	115	3
3.07	112, 112, 112, 111, 114	112	1	5		

Table A 88: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in test medium

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). The biological control samples, as well as analyzed analytical blanks showed no significant interference in the chromatogram at the retention time of prothioconazole. Any interference is clearly below 30 % of the lowest spike level. Typical chromatogram of an analytical control sample and a biological control sample are given for both mass transitions and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). The biological control samples, as well as analyzed analytical blanks showed no significant interference in the chromatogram at the retention time of azoxystrobin. Any interference is clearly below 30 % of the lowest spike level. Typical chromatogram of an analytical control sample and a biological control sample are given for both mass transitions and product ion spectra are provided.

	Prothioconazole	Azoxystrobin
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 performed with 1/x weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A89 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A89 below.
Calibration range	Linearity was confirmed over the calibration range 0.0405 – 4.19 µg/L (n = 8), covering test samples with a test item concentration of 0.0006 to 3.6 mg test item/L and thus covers the range from 6.0% of the LOQ to 16% above the highest spike (61% above the highest test concentration).	Linearity was confirmed over the calibration range 0.040 – 4.13 µg/L (n = 8), covering test samples with a test item concentration of 0.0006 to 3.5 mg test item/L and thus covers the range from 5.8% of the LOQ to 14% above the highest spike (56% above the highest test concentration).
Assessment of matrix effects is presented	Yes: Insignificant	Yes: Insignificant
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.00982 mg test item/L, corresponding to 1.36 µg prothioconazole/L. The LOD was derived from the lowest calibration level. The LOD is 0.08 µg prothioconazole/L.	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.00982 mg test item/L, corresponding to 1.38 µg azoxystrobin/L. The LOD was derived from the lowest calibration level. The LOD is 0.08 µg azoxystrobin/L.
Stability of standards and extracts	The investigation of the stability of standard solutions was not necessary, because these were analyzed within 24 hours after preparation. The stability of Prothioconazole was proven to be at least 51 days in the freezer ($\leq -15^{\circ}\text{C}$).	The investigation of the stability of standard solutions was not necessary, because these were analyzed within 24 hours after preparation. The stability of azoxystrobin was proven to be at least 51 days in the freezer ($\leq -15^{\circ}\text{C}$).

Table A 89: Linearity of detector response

Analyte	Transition	Linearity data
Prothioconazole	344 → 189 m/z (Quantification)	$y = 134311 x + 345$, $r = 0.9999$ (n = 8)
	344 → 154 m/z (Confirmation)	$y = 109789 x - 115$, $r = 0.9998$ (n = 8)
Azoxystrobin	404 → 372 m/z (Quantification)	$y = 1423235 x + 11342$, $r = 0.9995$ (n = 8)
	404 → 344 m/z (Quantification)	$y = 1112860 x + 9433$, $r = 0.9995$ (n = 8)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin. The Limit of Quantification was 0.00982 mg test item/L for all analytes (corresponding to 1.38 µg azoxystrobin/L and 1.36 µg prothioconazole/L).

A 2.1.1.2.5.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.5.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.6 Analytical method 6: Test medium

Comments of zRMS:	A method was successfully validated for determination of azoxystrobin and prothioconazole in test water samples. The LOQ is 0.0205 mg test item/L corresponding to 2.89 µg azoxystrobin /L and 2.84 µg prothioconazole /L. The acceptance criteria of the SANTE guideline for accuracy (mean recovery 70 to 120%) and precision (RSD ≤ 20%) were met. The method is fit for purpose.
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Reference:	KCP 5.1.2/18
Report	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L SC) – Acute toxicity to <i>Daphnia magna</i> in a 48-Hour immobilization test, Dupont A., 2023, Report N°20210196
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The test item concentration in the samples was determined by high pressure liquid chromatography (HPLC) with MS/MS detection using external calibration.

Immediately after sampling, acetonitrile was added to each sample to stabilize the latter during the storage period in frozen conditions. After storage period, the test samples were thawed at room temperature for about 1 hour. If necessary, the samples were further diluted into the calibration range with water/acetonitrile (1/1, v/v) mixture. The complete test sample work-up process took place under red light. An aliquot of the diluted samples was analyzed by HPLC with MS/MS detection. The limit of quantification was 0.0205 mg test item/L corresponding to 2.89 µg azoxystrobin/L and 2.84 µg prothioconazole/L.

Analytical conditions

Chromatographic conditions

System: 1290 Infinity II High speed pump, Agilent technologies
Autosampler: Agilent 1290 Infinity II Multisampler
Pre-column: Phenomenex SecurityGuard ULTRA C18
Column: Restek Pinnacle DB AQ C18 AQC18, 50 mm x 2.1 mm, 1.9 µm
Mobile phase A: 0.1% v/v formic acid in water
Mobile phase B: 0.1% v/v formic acid in acetonitrile
Flow: 0.6 mL/min
Column temperature: 40°C
Injection volume: 50 µL
Injection temperature: 10°C

Time (min)	% A	% B
0	95	5
0.5	70	30
3.0	10	90
4.0	10	90
4.1	95	5
5.0	95	5

Retention time:

Prothioconazole: About 2.5 min

Azoxystrobin: About 2.4 min

Mass spectrometric conditions

System: AB Sciex / QTRAP 6500
Ionisation type: Electrospray ionisation (ESI)
Polarity: Positive ion mode
Capillary voltage: 4500V QTRAP 6500
Source temperature: 500°C
Mass transitions

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation
Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 90: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 344 → 189 <i>m/z</i> (Proposed for Quantification)						
0.0205	97, 101, 102, 101, 100	100	2	5	93	9
6.04	79, 80, 90, 88, 95	86	8	5		
Transition <i>m/z</i> 344 → 154 <i>m/z</i> (Proposed for Quantification)						
0.0205	97, 104, 102, 101, 100	101	3	5	93	10
6.04	78, 81, 91, 88, 94	86	7	5		

Table A 91: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)						
0.0205	103, 103, 103, 103, 74	97	13	5	88	15
6.04	74, 73, 82, 81, 86	79	7	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Quantification)						
0.0205	101, 102, 104, 102, 74	97	13	5	88	15
6.04	74, 73, 81, 81, 87	79	7	5		

Table A 92: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in test medium

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the biological control sample and analytical blank. Representative chromatograms (lowest calibration level, control sample and samples fortified at the lowest fortification level) for both mass transitions and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the biological control sample and analytical blank. Representative chromatograms (lowest calibration level, control sample and samples fortified at the lowest fortification level) for both mass transitions 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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A93 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$) performed with 1/x weighting. The correlation coefficients, r obtained were > 0.99. Please see table A93 below.

	Prothioconazole	Azoxystrobin
Calibration range	Linearity was confirmed over the calibration range 0.0100 – 1.04 µg/L (n = 9), covering test samples with a test item concentration of 0.00014 to 7.54 mg test item/L and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.	Linearity was confirmed over the calibration range 0.00998 – 1.03 µg/L (n = 16), covering test samples with a test item concentration of 0.00014 to 7.34 mg test item/L and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract.
Assessment of matrix effects is presented	Yes (Unsignificant)	Yes (Unsignificant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.0205 mg test item/L, corresponding to 2.84 µg prothioconazole/L. The LOD was derived from the lowest calibration level. The LOD is 0.02 µg prothioconazole/L.	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.0205 mg test item/L, corresponding to 2.89 µg azoxystrobin/L. The LOD was derived from the lowest calibration level. The LOD is 0.02 µg azoxystrobin/L.
Stability of standards and extracts	The investigation of the stability of standard solutions and processed samples was not necessary, because these were analyzed within 24 hours after preparation.	The investigation of the stability of standard solutions and processed samples was not necessary, because these were analyzed within 24 hours after preparation.

Table A 93: Linearity of detector response

Analyte	Transition	Linearity data
Prothioconazole	344 → 189 m/z (Quantification)	y = 273406 x - 231, r = 0.9997 (n = 9)
	344 → 154 m/z (Confirmation)	y = 210265 x + 94.2, r = 0.9996 (n = 9)
Azoxystrobin	404 → 372 m/z (Quantification)	y = 4738054 x + 1512 r = 0.9953 (n = 16)
	404 → 344 m/z (Quantification)	y = 2394918 x + 996 r = 0.9992 (n = 16)

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in test water. The Limit of Quantification was 0.0205 mg test item/L for all analytes (corresponding to 2.89 µg azoxystrobin/L and 2.84 µg prothioconazole/L).

A 2.1.1.2.6.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.6.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.7 Method validation 7: Test medium

Comments of zRMS:	A method was successfully validated for determination of azoxystrobin and prothioconazole in test water samples. The Limit of Quantification was 0.0000445 mg test item/L (corresponding to 0.0627 µg azoxystrobin/L and 0.0617 µg prothioconazole/L). The acceptance criteria of the SANTE guideline for accuracy (mean recovery 70 to 120%) and precision ($RSD \leq 20\%$) were met. The method is fit for purpose.
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Reference:	KCP 5.1.2/19
Report	CA3642 (Azoxystrobin 150 g/L + Prothioconazole 150 g/L) – Effect on <i>Skeletonema</i> sp. In a 72-hour Algal growth-inhibition test, Dupont A., 2023, Report N°20210197
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The test item concentration in the samples was determined by high pressure liquid chromatography (HPLC) with MS/MS detection using external calibration.

Immediately after sampling, acetonitrile was added to each sample to stabilize the latter during the storage period in freezing conditions (at about -20°C).

The frozen test samples were thawed at room temperature for about 1 hour. The test and control samples from day 1, 2 and 3 were centrifuged (2465g, 5 min) due to the presence of algae. If necessary, the samples were diluted with water/acetonitrile (1/1, v/v) in order to be in calibration range. The complete test sample work-up process took place under red light. An aliquot of the diluted samples was analyzed by HPLC with MS/MS detection. The limit of quantification was 0.000445 mg test item/L corresponding to 0.0627 µg azoxystrobin/L and 0.0617 µg prothioconazole/L.

Analytical conditions

Chromatographic conditions

System: 1290 Infinity II High speed pump, Agilent technologies

Autosampler: Agilent 1290 Infinity II Multisampler

Pre-column: Phenomenex SecurityGuard ULTRA C18

Column: Waters Acquity UPLC C18, 50 mm x 2.1mm, 1.7µm

Mobile phase A: 0.1% v/v formic acid in water

Mobile phase B: 0.1% v/v formic acid in acetonitrile

Flow: 0.5 mL/min

Column temperature: 40°C

Injection volume: 50 µL

Injection temperature: 10°C

Time (min)	% A	% B
0	95	5
3.0	5	95
4.0	5	95
4.1	95	5
5.0	95	5

Retention time:

Prothioconazole: About 2.9 min

Azoxystrobin: About 2.8 min

Mass spectrometric conditions

System: AB Sciex / QTRAP 6500+

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Ion Spray voltage: 5000V QTRAP 6500+

Source temperature: 500°C

Mass transitions

Prothioconazole: 344 → 189 (m/z) for quantification, 344 → 154 (m/z) for confirmation

Azoxystrobin: 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 94: Recovery results from method validation of prothioconazole using the analytical method

Prothioconazole						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 344 → 189 <i>m/z</i> (Proposed for Quantification)						
0.000445	102; 92; 94; 94; 92	95	4	5	99	6
1.20	104; 100; 106; 104; 106	104	2	5		
Transition <i>m/z</i> 344 → 154 <i>m/z</i> (Proposed for Quantification)						
0.000445	90; 92; 92; 87; 96	91	4	5	97	6
1.20	102; 103; 104; 97; 103	102	3	5		

Table A 95: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin						
Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)						
0.000445	79; 85; 78; 79; 71	78	6	5	81	9
1.20	88; 80; 76; 81; 96	84	9	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Quantification)						
0.000445	78; 81; 78; 79; 67	77	7	5	80	9
1.20	88; 80; 77; 78; 96	84	10	5		

Table A 96: Characteristics for the analytical method used for validation of prothioconazole and azoxystrobin in test medium

	Prothioconazole	Azoxystrobin
Specificity	MS/MS determination was conducted by monitoring two mass transitions (m/z 344 → 189 for quantification and m/z 344 → 154 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the biological control sample and analytical blank. Representative chromatograms (lowest calibration level, control sample and samples fortified at the lowest fortification level) for both mass transitions and product ion spectra are provided.	MS/MS determination was conducted by monitoring two mass transitions (m/z 404 → 372 for quantification and m/z 404 → 344 for confirmation). No interference at the retention time of Prothioconazole above 30% of the LOQ were observed in the biological control sample and analytical blank. Representative chromatograms (lowest calibration level, control sample and samples fortified at the lowest fortification level) for both mass transitions 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 performed with 1/x ² weighting (regression model: y = a*x + b). The correlation coefficients, r obtained were > 0.99. Please see table A97 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression performed with 1/x ² weighting analysis (regression model: y = a*x + b). The correlation coefficients, r obtained were > 0.99. Please see table A97 below.
Calibration range	Linearity was confirmed over the calibration range 0.0100 – 1.04 µg/L (n = 9), covering test	Linearity was confirmed over the calibration range 0.00999 – 1.033 µg/L (n = 9), covering test

	Prothioconazole	Azoxystrobin
	samples with a test item concentration of 0.00014 to 1.5 mg test item/L and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract.	samples with a test item concentration of 0.00014 to 1.5 mg test item/L and thus covers the range from no more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in any (diluted) sample extract.
Assessment of matrix effects is presented	Yes (significant)	Yes (significant)
Limit of determination/quantification	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.000445 mg test item/L, corresponding to 0.0617 µg prothioconazole/L. The LOD was derived from the lowest calibration level. The LOD is 0.02 µg prothioconazole/L (taking into account a sample dilution factor of 2).	The LOQ was derived from the lowest spike level which gives acceptable data for recovery and precision. The LOQ is 0.000445 mg test item/L, corresponding to 0.0627 µg azoxystrobin/L. The LOD was derived from the lowest calibration level. The LOD is 0.02 µg azoxystrobin/L (taking into account a sample dilution factor of 2).
Stability of standards and extracts	The investigation of the stability of standard solutions and processed samples was not necessary, because these were analyzed within 24 hours after preparation.	The investigation of the stability of standard solutions and processed samples was not necessary, because these were analyzed within 24 hours after preparation.

Table A 97: Linearity of detector response

Analyte	Transition	Linearity data
Prothioconazole	344 → 189 m/z (Quantification)	$y = 390505 x + 411, r = 0.9957 (n = 9)$
	344 → 154 m/z (Confirmation)	$y = 353277 x + 795, r = 0.9966 (n = 9)$
Azoxystrobin	404 → 372 m/z (Quantification)	$y = 7214597 x + 4414 r = 0.9950 (n = 9)$
	404 → 344 m/z (Quantification)	$y = 3913558 x + 2992 r = 0.9943 (n = 9)$

Conclusion

The method validation is considered valid and acceptable for specificity, linearity, accuracy and precision according SANTE/2020/12830 rev.1 for the determination of prothioconazole and azoxystrobin in test water. The Limit of Quantification was 0.0000445 mg test item/L (corresponding to 0.0627 µg azoxystrobin/L and 0.0617 µg prothioconazole/L).

A 2.1.1.2.7.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.1.2.7.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.1.2.8 Analytical method – Determination of prothioconazole and azoxystrobin in acetonitrile rinse and tank mix solutions (Effectiveness of cleaning study)

A 2.1.1.2.8.1 Method validation

Comments of zRMS:	The method is sufficiently described and is considered fit for purpose.
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Reference:	KCP 5.1.2/23
Report	CA3642 – Effectiveness of Cleaning, Calvert A., 2023, 23/1610
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 25 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: Ace 5, C18, 250 x 4.6 mm

Mobile phase: 45/55 v/v, Acetonitrile: 0.1% v/v Phosphoric Acid

Flow rate: 1.0 mL/min

Column temperature: 30°C

Injection volume: 10 µL

Detector Wavelength: 250 nm

Results and discussions

Table A 9: 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	81.5	4.10
		400	97.4	1.35
	Azoxystrobin	0.5	100.0	0.33
		400	97.7	1.14

Table A 10: Characteristics for the analytical method used for validation of residues in acetonitrile rinse and tank mix solutions

	Prothioconazole	Azoxystrobin
Specificity	Blank solutions of tap water, hardwater D, acetonitrile and 2.3 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.	Blank solutions of tap water, hardwater D, acetonitrile and 2.3 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: $y =$	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: $y =$

	Prothioconazole	Azoxystrobin
	$311.579364 \times (n=5)$ $r = 1.0000$	$361.832663 \times (n=5)$ $r = 1.0000$
Calibration range	0.0004 – 0.5 mg/mL (corresponding to 0.4 to 500 mg/L)	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.	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)	LOQ = 0.5 mg azoxystrobin/L LOD = 0.4 mg azoxystrobin/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.	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 and azoxystrobin in acetonitrile rinse and tank mix solutions at an LOQ of 0.5 mg/L.

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)

A 2.1.2.1.1 Azoxystrobin and metabolites in different matrix groups (high water content, high oil content and high protein/high starch content)

A 2.1.2.1.1.1 Method validation

Comments of zRMS:	<p>The method has been successfully validated according to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4 for the determination of residues of azoxystrobin and its Z-isomer in different plant matrices (32 plant material matrices) including stability of azoxystrobin and its Z-isomer in extracts from selected matrices with the LOQ of 0.01 mg/kg. A highly specific detection system was used (MS/MS). Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes and matrices at each level.</p> <p>The acceptance criteria of the SANTE/2020/12830 rev.2 for analytical method were met.</p> <p>The method is acceptable.</p>
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Reference:	KCP 5.2/01
Report	<p>Final report and Supplement A to final report</p> <p>AZOXYSTROBIN: Validation of residue analytical method and storage stability of residue during storage of sample, Kawa-Miszczak L., 2011, report number PBBZ-2011/07/DPL</p>
Guideline(s):	<p>SANCO/825/00 rev. 8.1</p> <p>SANCO/3029/99 rev. 4</p>
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Samples of oilseed rape were extracted with acetonitrile/water in the presence of hexane, after addition of buffer salt mixture. The acetonitrile phase was then extracted twice by QuEChERS method (50mg PSA, 50mg DSC-18 and 50mg magnesium sulfate). An aliquot of 0.5mL of extract was diluted with water and acetonitrile prior to LC-MS/MS determination.

Samples of summer barley, winter wheat, green beans and rotational crop matrices were extracted with acetonitrile after addition of water (if necessary) in the presence buffer salt mixture. The acetonitrile phase was then extracted by QuEChERS method (38mg PSA, 225mg magnesium sulfate). An aliquot of 0.5mL of extract was diluted with water and acetonitrile prior to analyse. Azoxystrobin and Azoxystrobin-Z-isomer were analysed by LC-MS/MS. Matrix-matched standards were used for quantitation. The intended limit of quantification (LOQ) was 0.01 mg/kg in all matrix types for Azoxystrobin and Azoxystrobin-Z-isomer.

Analytical conditions

LC conditions

System: 1200 Series, Agilent Technologies

Degasser G1379B

Bin Pump SL G1312B

HiP – ALS SL G1367 C

FC-ALS Therm

TCC SL G1316B (thermostated column compartment)

Column: Zorbax XDB-C18, 50 mm x 4.6 mm, 1.8µm

Column temperature: 40°C

Mobile phase

Eluent A: water/acetonitrile/acetic acid (4.5/0.5/(0.2% in water) v/v)

Eluent B: water/acetonitrile (0.5/4.5 v/v)

Gradient

Time (min)	% A	% B
0	50	50
10	50	50

Flow: 0.4 L/min

Injection volume: 5 µL // 10 µL

Retention time:

Azoxystrobin: About 6.3 min

Azoxystrobin-Z-isomer: About 4.8 min

MS conditions

System: Triple quadrupole G6410A

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive

Drying gas Temperature: 350°C

Drying gas flow: 9L/min

Nebulizer: 40 psi

Capillary voltage (IS) 4000 V

Compound name	Precursor ion (m/z)	Product ion (m/z)	Fragmentor
Azoxystrobin-Z	404.1	372.2 (P)	85
Azoxystrobin-Z	404.1	344.2	85
Azoxystrobin-Z	404.1	329.1	85
Azoxystrobin-Z	404.1	172.1	85
Azoxystrobin	404.1	372.2 (P)	112
Azoxystrobin	404.1	344.2	112
Azoxystrobin	404.1	329.1	112
Azoxystrobin	404.1	172.1	112

Results and discussions

Table A 98: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 (Proposed for Quantification)							
Oilseed rape (grain)	0.01	94.8, 93.8, 91.0, 93.3, 99.9	94.5	3.48	5	94.4	2.70
	0.1	91.7, 96.2, 93.6, 96.0, 93.2	94.2	2.03	5		
Oilseed rape (cake)	0.01	93.9, 105.7, 97.1, 107.7, 98.8	100.6	5.80	5	95.5	7.24
	0.1	93.4, 90.5, 86.1, 92.3, 89.7	90.4	3.12	5		
Oilseed rape (oil)	0.01	93.4, 98.2, 96.1, 99.8, 96.6	96.8	2.49	5	94.5	3.62
	0.1	93.7, 94.0, 87.9, 94.1, 91.3	92.2	2.88	5		
Barley (grain)	0.01	104.8, 95.7, 125.3, 99.7, 100.7	105.3	11.09	5	106.6	7.46
	0.1	107.5, 107.3, 109.8, 108.2, 106.9	107.9	1.04	5		
Summer barley (straw)	0.01	97.8, 100.2, 105.2, 102.4, 103.3	101.8	2.80	5	95.3	7.58
	0.1	86.7, 88.0, 92.7, 88.1, 88.8	88.9	2.54	5		
Barley (malt)	0.01	104.4, 108.2, 107.3, 107.4, 111.4	107.7	2.30	5	104.2	5.73
	0.1	104.4, 105.6, 98.6, 90.1, 104.7	100.7	6.49	5		
Barley (wort)	0.01	106.4, 102.4, 104.0, 99.1,	102.2	3.15	5	100.3	3.07

		98.8					
	0.1	96.7, 101.1, 98.7, 98.6, 97.6	98.5	1.69	5		
Barley (germs)	0.01	111.0, 109.2, 86.2, 101.9, 103.7	102.4	9.54	5	98.4	8.44
	0.1	93.2, 101.0, 94.1, 88.4, 95.7	94.5	4.81	5		
Barley (spent grains)	0.01	104.3, 104.5, 97.6, 101.4, 100.3	101.6	2.88	5	103.8	4.51
	0.1	101.1, 104.8, 114.0, 108.6, 101.6	106.0	5.05	5		
Barley (yeast)	0.01	75.5, 66.1, 92.6, 101.1, 70.8	81.2	18.40	5	86.0	13.14
	0.1	88.2, 93.7, 91.7, 92.3, 88.2	90.8	2.77	5		
Barley (waste)	0.01	101.8, 98.6, 97.0, 103.4, 104.2	101.0	3.05	5	100.3	2.87
	0.1	98.3, 96.7, 97.8, 102.7, 102.6	99.6	2.83	5		
Barley (beer)	0.01	82.0, 93.9, 88.9, 85.3, 91.9	88.4	5.45	5	90.3	4.53
	0.1	89.0, 95.2, 92.4, 92.1, 92.7	92.3	2.42	5		
Barley (pot barley)	0.01	104.5, 107.3, 103.7, 103.5, 102.1	104.2	1.85	5	108.5	4.85
	0.1	114.1, 117.0, 110.0, 108.5, 114.6	112.9	3.09	5		
Barley (hulls)	0.01	84.0, 95.1, 92.0, 85.9, 74.3	86.3	9.35	5	95.9	12.79
	0.1	116.3, 105.8, 102.2, 104.0, 95.5	105.6	6.10	5		
Winter wheat (green material)	0.01	104.1, 113.4, 111.0, 112.5, 108.8	110.0	3.37	5	108.8	3.13
	0.1	109.8, 108.2, 105.0, 110.9, 104.0	107.6	2.76	5		
Winter wheat (grain)	0.01	112.8, 106.9, 106.5, 110.4, 93.8	106.1	6.94	5	105.9	4.70
	0.1	104.4, 106.8, 106.6, 104.3, 106.3	105.7	1.31	5		
Winter wheat (straw)	0.01	111.3, 111.1, 106.0, 105.7, 109.9	108.8	2.53	5	102.1	7.78
	0.1	89.1, 93.7, 101.9, 96.9, 95.3	95.4	4.88	5		
Winter wheat (water)	0.01	105.5, 111.2, 108.4, 104.2, 99.7	105.8	4.12	5	99.6	7.58
	0.1	95.1, 94.6, 92.3, 97.3, 87.7	93.4	3.88	5		
Winter wheat (bran)	0.01	96.8, 102.2, 91.6, 98.7, 94.0	96.7	4.25	5	97.7	6.78
	0.1	91.6, 91.2, 95.9, 103.0, 112.2	98.8	9.00	5		
Winter wheat (middling)	0.01	111.6, 105.2, 99.2, 108.9, 105.9	106.2	4.39	5	107.9	3.51
	0.1	108.1, 111.6, 108.8, 111.3, 109.0	109.7	1.43	5		
Winter wheat (flour)	0.01	100.7, 109.1, 111.4, 116.8, 106.0	108.8	5.51	5	109.8	4.05
	0.1	111.0, 113.5, 111.0, 111.6, 106.8	110.8	2.24	5		
Winter wheat (dough)	0.01	108.8, 108.5, 113.8, 89.7, 93.4	102.8	10.31	5	106.5	7.58
	0.1	109.5, 109.3, 109.6, 111.5, 110.8	110.1	0.86	5		
Winter wheat (bread)	0.01	100.1, 90.9, 112.0, 112.7, 111.4	105.4	9.14	5	108.7	7.19
	0.1	104.3, 113.8, 113.1, 114.2, 114.2	111.9	3.82	5		
Winter wheat (germs)	0.01	110.4, 109.9, 103.6, 107.8, 105.5	107.4	2.67	5	100.8	7.26
	0.1	95.1, 94.6, 95.4, 94.8, 91.1	94.2	1.86	5		
Green beans (plants)	0.01	95.3, 115.8, 112.6, 111.4, 106.4	108.3	7.39	5	108.7	5.52
	0.1	102.5, 108.5, 112.9, 109.2, 112.0	109.0	3.74	5		
Green beans (pods)	0.01	93.4, 91.2, 90.5, 94.3, 88.9	91.6	2.38	5	95.7	4.88
	0.1	96.6, 101.4, 100.8, 100.8,	99.7	2.00	5		

		98.7					
Green beans (tips)	0.01	98.3, 94.4, 101.1, 104.4, 101.5	99.9	3.77	5	96.4	4.86
	0.1	92.4, 91.3, 94.1, 95.5, 90.9	92.8	2.11	5		
Green beans (cooked beans)	0.01	107.5, 107.3, 106.8, 107.8, 105.6	107.0	0.79	5	104.1	3.04
	0.1	99.6, 101.1, 102.8, 101.5, 101.0	101.2	1.13	5		
Rotational crop: Barley/ green material	0.01	108.9, 99.0, 97.8, 94.6, 93.4	98.7	6.18	5	99.2	4.34
	0.1	99.1, 100.1, 101.9, 100.7, 96.7	99.7	1.99	5		
Rotational crop: Radish / roots	0.01	96.8, 98.1, 99.0, 100.3, 100.8	99.0	1.63	5	100.9	3.05
	0.1	98.2, 100.5, 104.3, 105.0, 105.5	102.7	3.08	5		
Rotational crop: Radish / leaves	0.01	106.1, 109.5, 109.6, 108.4, 102.3	107.2	2.88	5	107.8	2.89
	0.1	105.6, 108.9, 108.3, 113.8, 105.6	108.5	3.09	5		
Rotational crop: Lettuce / heads	0.01	111.8, 110.1, 112.4, 109.7, 106.2	110.0	2.20	5	109.9	2.75
Transition m/z 404 → 329 (Proposed for Quantification)*							
Oilseed rape (grain)	0.01	91.5, 83.0, 93.5, 99.2, 100.5	93.5	7.50	5	93.2	5.39
	0.1	89.5, 96.5, 91.2, 93.2, 94.0	92.9	2.89	5		
Oilseed rape (cake)	0.01	78.8, 102.7, 90.5, 104.5, 95.6	94.4	10.98	5	93.6	7.85
	0.1	96.6, 93.6, 87.1, 93.4, 93.1	92.8	3.76	5		
Oilseed rape (oil)	0.01	92.9, 98.0, 94.8, 102.5, 103.5	98.3	4.74	5	96.5	4.13
	0.1	97.2, 96.0, 90.8, 95.1, 94.5	94.7	2.55	5		
Barley (grain)	0.01	99.4, 103.3, 121.9, 99.3, 95.3	103.8	10.09	5	106.4	7.14
	0.1	109.7, 111.9, 108.0, 108.2, 107.0	109.0	1.75	5		
Summer barley (straw)	0.01	104.0, 95.9, 103.0, 104.5, 94.2	100.3	4.88	5	94.6	7.34
	0.1	86.7, 87.6, 91.8, 88.6, 90.1	89.0	2.28	5		
Barley (malt)	0.01	106.7, 112.1, 110.4, 107.1, 112.4	109.7	2.48	5	105.4	6.03
	0.1	103.9, 106.9, 98.2, 91.9, 104.3	101.0	5.96	5		
Barley (wort)	0.01	109.5, 121.2, 115.3, 117.9, 104.9	113.7	5.75	5	106.3	8.42
	0.1	98.1, 100.0, 99.0, 98.4, 99.1	98.9	0.73	5		
Barley (germs)	0.01	110.8, 117.7, 95.8, 112.8, 101.6	107.8	8.22	5	100.7	10.08
	0.1	92.7, 101.7, 92.3, 87.2, 93.9	93.6	5.60	5		
Barley (spent grains)	0.01	95.3, 118.7, 116.1, 106.7, 94.7	106.3	10.55	5	107.9	7.96
	0.1	106.7, 108.7, 118.5, 110.9, 102.9	109.5	5.31	5		
Barley (yeast)	0.01	73.9, 70.3, 95.6, 101.3, 75.4	83.3	16.94	5	88.2	12.28
	0.1	91.3, 93.1, 93.4, 96.6, 91.0	93.1	2.41	5		
Barley (waste)	0.01	109.7, 96.7, 88.9, 89.9, 101.4	97.3	8.84	5	97.7	6.17
	0.1	98.8, 94.9, 95.5, 100.3, 100.7	98.0	2.76	5		
Barley (beer)	0.01	74.3, 77.6, 79.6, 74.3, 78.6	76.9	3.21	5	84.7	9.95
	0.1	93.5, 94.3, 91.3, 90.7, 92.4	92.4	1.59	5		
Barley (pot barley)	0.01	94.1, 105.6, 92.5, 95.4, 90.3	95.6	6.21	5	103.0	8.81
	0.1	113.0, 112.5, 105.1, 108.9, 113.0	110.5	3.14	5		
Barley (hulls)	0.01	91.8, 107.8, 106.2, 90.5, 87.4	96.7	9.81	5	101.2	8.60
	0.1	114.9, 105.6, 102.0, 104.9, 101.1	105.7	5.20	5		

Winter wheat (green material)	0.01	107.4, 115.5, 114.8, 115.4, 107.4	112.1	3.83	5	109.3	4.29
	0.1	109.3, 106.3, 103.6, 110.4, 102.8	106.5	3.17	5		
Winter wheat (grain)	0.01	115.6, 87.1, 108.0, 113.6, 114.9	107.8	11.13	5	107.7	7.56
	0.1	103.8, 107.6, 108.3, 108.3, 109.8	107.6	2.08	5		
Winter wheat (straw)	0.01	125.4, 111.4, 120.3, 109.1, 112.1	115.7	5.98	5	107.1	9.78
	0.1	94.2, 96.3, 105.0, 99.9, 97.6	98.6	4.21	5		
Winter wheat (water)	0.01	107.0, 115.6, 114.7, 116.1, 104.3	111.6	4.92	5	104.1	8.59
	0.1	99.8, 95.5, 91.4, 98.9, 97.8	96.7	3.50	5		
Winter wheat (bran)	0.01	90.3, 95.6, 87.5, 87.1, 77.8	87.7	7.38	5	90.8	10.98
	0.1	84.2, 85.9, 88.9, 95.4, 115.0	93.9	13.35	5		
Winter wheat (middling)	0.01	109.9, 105.7, 100.8, 111.8, 95.1	104.6	6.52	5	107.0	5.00
	0.1	107.7, 112.1, 107.7, 110.3, 109.3	109.4	1.75	5		
Winter wheat (flour)	0.01	97.0, 107.0, 105.2, 115.3, 105.6	106.0	6.12	5	111.1	6.50
	0.1	114.0, 114.6, 113.9, 116.4, 121.9	116.2	2.89	5		
Winter wheat (dough)	0.01	103.2, 107.2, 137.4, 88.8, 102.9	107.9	16.58	5	109.6	11.02
	0.1	111.1, 113.0, 110.5, 111.5, 110.4	111.3	0.94	5		
Winter wheat (bread)	0.01	98.9, 112.3, 117.7, 104.1, 117.2	110.1	7.51	5	112.0	6.58
	0.1	102.2, 117.0, 116.6, 114.7, 118.9	113.9	5.87	5		
Winter wheat (germs)	0.01	111.9, 109.0, 104.8, 110.6, 102.4	107.7	3.72	5	101.9	6.63
	0.1	96.5, 98.2, 96.1, 95.6, 94.2	96.1	1.54	5		
Green beans (plants)	0.01	101.2, 115.0, 112.6, 113.0, 106.2	109.6	5.23	5	111.5	4.02
	0.1	112.7, 110.8, 115.4, 113.6, 114.7	113.4	1.57	5		
Green beans (pods)	0.01	92.5, 89.3, 86.4, 89.6, 89.7	89.5	2.41	5	95.5	6.87
	0.1	99.6, 102.3, 102.5, 102.6, 100.3	101.5	1.39	5		
Green beans (tips)	0.01	98.7, 97.5, 103.5, 104.7, 102.4	101.4	3.08	5	97.0	5.32
	0.1	92.2, 91.5, 93.4, 94.6, 91.2	92.6	1.51	5		
Green beans (cooked beans)	0.01	103.5, 110.7, 109.6, 108.0, 105.9	107.5	2.69	5	104.5	3.59
	0.1	100.6, 101.4, 102.1, 101.4, 101.7	101.4	0.54	5		
Rotational crop: Barley/ green material	0.01	102.4, 98.9, 96.5, 88.4, 86.1	94.4	7.37	5	96.8	5.74
	0.1	98.3, 99.2, 102.3, 100.7, 95.4	99.2	2.63	5		
Rotational crop: Radish / roots	0.01	91.5, 101.0, 96.5, 101.8, 98.4	97.8	4.25	5	99.9	4.00
	0.1	98.1, 101.2, 104.8, 101.3, 104.6	102.0	2.75	5		
Rotational crop: Radish / leaves	0.01	111.3, 113.3, 114.4, 119.2, 105.4	112.7	4.44	5	110.9	3.58
	0.1	108.2, 111.3, 110.1, 107.6, 108.4	109.1	1.40	5		
Rotational crop: Lettuce / heads	0.01	108.0, 108.4, 113.7, 108.1, 109.5	109.5	2.17	5	109.4	3.04
	0.1	107.1, 109.4, 103.8, 115.7, 110.1	109.2	4.01	5		

* KCP 5.2/01b Report PBBZ-2011-07-DPL Supplement

Table A 99: Recovery results from method validation of azoxystrobin-Z-isomer using the analytical method

Azoxystrobin-Z-isomer							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 (Proposed for Quantification)							
Oilseed rape (grain)	0.01	100.5, 100.6, 98.3, 94.4, 98.0	98.4	2.55	5	95.7	3.64
	0.1	90.4, 94.3, 94.8, 93.0, 92.5	93.0	1.86	5		
Oilseed rape (cake)	0.01	95.5, 102.4, 100.7, 101.8, 98.7	99.8	2.78	5	95.5	5.43
	0.1	91.3, 92.6, 87.3, 93.1, 91.2	91.1	2.49	5		
Oilseed rape (oil)	0.01	100.7, 95.8, 96.0, 99.3, 100.4	98.4	2.39	5	96.1	3.52
	0.1	96.1, 95.5, 89.8, 95.2, 92.6	93.8	2.81	5		
Barley (grain)	0.01	98.3, 91.6, 100.9, 94.0, 96.2	96.2	3.76	5	101.4	5.95
	0.1	107.4, 107.2, 107.7, 106.3, 103.8	106.5	1.48	5		
Summer barley (straw)	0.01	90.5, 89.3, 93.7, 95.9, 93.0	92.5	2.85	5	90.4	3.58
	0.1	85.4, 87.5, 92.0, 88.3, 88.2	88.3	2.68	5		
Barley (malt)	0.01	109.9, 109.9, 106.2, 108.2, 109.3	108.7	1.45	5	104.0	6.80
	0.1	102.4, 104.7, 96.6, 87.5, 105.2	99.3	7.47	5		
Barley (wort)	0.01	100.0, 96.7, 102.3, 96.5, 102.9	99.7	3.05	5	98.5	2.69
	0.1	95.7, 99.7, 96.3, 98.3, 96.2	97.2	1.74	5		
Barley (germs)	0.01	94.3, 90.4, 78.7, 89.7, 93.6	89.3	7.02	5	92.2	6.23
	0.1	94.4, 100.9, 95.6, 90.5, 94.0	95.1	3.98	5		
Barley (spent grains)	0.01	104.9, 103.7, 95.1, 100.4, 94.9	99.8	4.69	5	103.8	6.30
	0.1	102.6, 104.5, 116.5, 111.1, 104.2	107.8	5.45	5		
Barley (yeast)	0.01	96.6, 82.6, 85.9, 107.6, 91.8	92.9	10.58	5	92.3	7.47
	0.1	87.2, 92.6, 95.0, 93.4, 0.1	92.3	7.47	5		
Barley (waste)	0.01	100.8, 99.5, 98.0, 107.2, 104.7	102.0	3.74	5	100.6	3.55
	0.1	98.3, 96.4, 96.8, 101.6, 103.3	99.3	3.08	5		
Barley (beer)	0.01	94.6, 94.9, 96.9, 92.4, 99.9	95.8	2.94	5	94.5	2.88
	0.1	91.1, 96.9, 92.6, 93.3, 92.4	93.3	2.36	5		
Barley (pot barley)	0.01	108.0, 111.9, 108.2, 103.9, 105.9	107.6	2.78	5	109.0	2.73
	0.1	110.1, 113.1, 109.1, 107.2, 112.5	110.4	2.21	5		
Barley (hulls)	0.01	107.9, 116.3, 109.7, 105.4, 93.5	106.6	7.85	5	107.0	6.51
	0.1	118.1, 106.8, 103.5, 105.9, 102.6	107.4	5.79	5		
Winter wheat (green material)	0.01	98.9, 109.6, 107.6, 110.5, 105.8	106.5	4.33	5	106.8	3.58
	0.1	109.6, 107.8, 103.5, 110.9, 103.6	107.1	3.16	5		
Winter wheat (grain)	0.01	104.2, 101.7, 103.1, 107.1, 95.4	102.3	4.25	5	104.0	3.39
	0.1	104.0, 106.5, 105.7, 104.6, 107.5	105.7	1.31	5		
Winter wheat (straw)	0.01	100.2, 103.0, 104.0, 100.4, 102.2	102.0	1.59	5	98.3	4.85
	0.1	90.1, 92.3, 98.9, 98.1, 93.5	94.6	4.03	5		
Winter wheat (water)	0.01	108.6, 107.0, 104.3, 103.4, 103.5	105.4	2.19	5	99.6	6.58
	0.1	93.6, 95.6, 94.4, 96.1, 89.2	93.8	2.92	5		

Winter wheat (bran)	0.01	95.9, 98.0, 96.1, 108.2, 93.7	98.4	5.79	5	97.4	5.67
	0.1	90.6, 91.3, 95.2, 101.0, 103.7	96.4	6.02	5		
Winter wheat (middling)	0.01	108.4, 106.6, 104.4, 112.2, 112.4	108.8	3.23	5	108.4	2.50
	0.1	106.4, 109.7, 106.2, 110.5, 107.4	108.1	1.81	5		
Winter wheat (flour)	0.01	96.9, 105.3, 103.8, 113.2, 101.4	104.1	5.75	5	108.0	5.30
	0.1	112.6, 113.1, 111.2, 111.6, 110.4	111.8	0.98	5		
Winter wheat (dough)	0.01	110.7, 111.1, 117.5, 93.7, 94.5	105.5	10.20	5	107.5	7.01
	0.1	109.3, 109.0, 110.9, 110.7, 107.7	109.5	1.19	5		
Winter wheat (bread)	0.01	103.8, 93.9, 113.4, 110.6, 114.4	107.2	7.94	5	109.5	6.14
	0.1	105.2, 111.1, 115.0, 114.9, 112.9	111.8	3.59	5		
Winter wheat (germs)	0.01	101.5, 97.4, 97.1, 97.7, 98.4	98.4	1.83	5	96.0	3.08
	0.1	94.9, 93.2, 94.6, 93.8, 91.4	93.6	1.47	5		
Green beans (plants)	0.01	95.4, 111.9, 109.5, 111.3, 107.3	107.1	6.33	5	108.2	5.07
	0.1	102.0, 108.8, 112.6, 110.8, 112.2	109.3	3.95	5		
Green beans (pods)	0.01	93.8, 91.0, 90.8, 94.3, 90.1	92.0	2.07	5	96.1	4.90
	0.1	97.3, 102.0, 101.4, 101.4, 99.2	100.3	1.97	5		
Green beans (tips)	0.01	100.4, 97.3, 102.0, 95.7, 99.0	98.9	2.49	5	95.7	3.99
	0.1	92.3, 91.8, 94.1, 93.8, 90.8	92.6	1.52	5		
Green beans (cooked beans)	0.01	108.4, 109.9, 108.3, 108.9, 105.3	108.2	1.61	5	104.1	4.34
	0.1	98.9, 99.8, 101.0, 100.3, 99.8	100.0	0.80	5		
Rotational crop: Barley / green material	0.01	88.9, 94.5, 95.8, 94.5, 90.3	92.8	3.22	5	95.5	4.05
	0.1	97.5, 98.3, 100.4, 100.6, 94.5	98.2	2.53	5		
Rotational crop: Radish / roots	0.01	97.9, 97.5, 97.2, 99.3, 100.6	98.5	1.45	5	100.5	2.63
	0.1	100.0, 101.9, 102.5, 103.6, 104.9	102.6	1.82	5		
Rotational crop: Radish / leaves	0.01	104.0, 109.1, 108.8, 110.8, 104.0	107.4	2.92	5	107.4	2.74
	0.1	104.7, 108.7, 106.4, 112.4, 105.4	107.5	2.89	5		
Rotational crop: Lettuce / heads	0.01	110.2, 110.6, 111.5, 109.2, 106.2	109.6	1.84	5	109.9	2.78
	0.1	107.9, 111.8, 104.7, 115.5, 110.8	110.2	3.71	5		
Transition m/z 404 → 329 (Proposed for Confirmation)*							
Oilseed rape (grain)	0.01	92.7, 103.7, 104.5, 102.4, 110.6	102.8	6.30	5	99.5	5.69
	0.1	93.9, 98.3, 96.6, 97.3, 94.9	95.2	1.87	5		
Oilseed rape (cake)	0.01	92.9, 103.9, 87.2, 98.0, 93.4	95.1	6.55	5	94.5	4.78
	0.1	95.2, 95.1, 89.6, 96.1, 94.0	94.0	2.71	5		
Oilseed rape (oil)	0.01	96.4, 92.1, 97.0, 91.4, 102.0	95.8	4.45	5	95.3	3.62
	0.1	98.2, 95.1, 91.1, 96.6, 92.9	94.8	2.99	5		
Barley (grain)	0.01	92.5, 108.7, 95.3, 118.3, 104.7	103.9	10.03	5	106.8	7.19
	0.1	110.0, 111.5, 110.0, 110.2, 106.4	109.6	1.72	5		
Summer barley (straw)	0.01	100.5, 95.0, 106.7, 92.7, 94.0	97.8	5.92	5	93.7	6.41
	0.1	87.0, 88.1, 93.0, 89.4, 90.3	89.6	2.56	5		
Barley (malt)	0.01	109.0, 116.4, 115.3, 110.1, 108.9	111.9	3.27	5	105.6	7.89

	0.1	102.5, 104.3, 96.8, 88.9, 104.0	99.3	6.61	5		
Barley (wort)	0.01	97.6, 118.3, 106.6, 130.6, 114.4	113.5	10.93	5	105.6	11.25
	0.1	97.3, 101.9, 98.0, 96.1, 95.0	97.7	2.69	5		
Barley (germs)	0.01	89.4, 101.2, 84.2, 83.9, 102.5	92.2	9.79	5	95.0	7.81
	0.1	97.7, 105.3, 98.9, 93.2, 94.2	97.9	4.87	5		
Barley (spent grains)	0.01	104.6, 111.3, 108.0, 112.3, 117.1	110.7	4.26	5	108.5	5.45
	0.1	100.6, 104.1, 116.5, 109.6, 101.1	106.4	6.29	5		
Barley (yeast)	0.01	86.8, 73.7, 78.4, 100.5, 68.4	81.6	15.35	5	85.3	10.89
	0.1	86.6, 89.9, 88.9, 89.4, 90.5	89.0	1.67	5		
Barley (waste)	0.01	98.3, 93.2, 99.7, 103.2, 101.1	99.1	3.81	5	99.5	4.25
	0.1	97.2, 95.1, 96.2, 105.0, 105.6	99.8	5.08	5		
Barley (beer)	0.01	87.0, 107.4, 116.6, 73.7, 112.6	99.4	18.46	5	96.0	13.36
	0.1	91.9, 93.8, 90.1, 94.2, 92.5	92.5	1.75	5		
Barley (pot barley)	0.01	110.6, 109.7, 115.1, 87.7, 112.5	107.1	10.31	5	108.5	7.14
	0.1	109.0, 113.9, 106.2, 109.0, 111.3	109.9	2.64	5		
Barley (hulls)	0.01	96.5, 129.8, 107.6, 100.0, 87.0	104.2	15.45	5	106.4	11.03
	0.1	118.9, 109.4, 104.5, 106.3, 103.6	108.5	5.72	5		
Winter wheat (green material)	0.01	105.2, 108.4, 106.8, 114.0, 106.7	108.2	3.17	5	107.6	2.85
	0.1	109.3, 107.2, 104.4, 110.0, 103.5	106.9	2.68	5		
Winter wheat (grain)	0.01	108.8, 107.3, 106.4, 105.0, 104.2	106.3	1.71	5	107.0	2.29
	0.1	103.1, 109.6, 110.7, 108.7, 106.5	107.7	2.78	5		
Winter wheat (straw)	0.01	107.3, 121.1, 97.1, 99.2, 99.3	104.5	9.44	5	103.1	7.36
	0.1	94.6, 100.9, 105.1, 107.0, 99.1	101.4	4.86	5		
Winter wheat (water)	0.01	105.0, 105.9, 123.2, 118.1, 108.5	112.1	7.19	5	104.0	9.83
	0.1	97.4, 96.8, 96.8, 96.4, 92.0	95.9	2.30	5		
Winter wheat (bran)	0.01	72.1, 89.9, 93.7, 102.6, 96.6	91.0	12.70	5	91.9	11.40
	0.1	87.4, 83.8, 89.9, 92.0, 110.9	92.8	11.37	5		
Winter wheat (middling)	0.01	95.6, 110.6, 102.3, 103.4, 111.4	104.7	6.24	5	105.7	4.44
	0.1	106.2, 107.0, 106.1, 109.9, 104.2	106.7	1.95	5		
Winter wheat (flour)	0.01	116.6, 127.9, 106.8, 112.2, 114.1	115.5	6.75	5	114.5	4.68
	0.1	114.3, 113.2, 112.4, 112.7, 114.7	113.4	0.90	5		
Winter wheat (dough)	0.01	93.7, 111.2, 110.9, 72.2, 78.9	93.4	19.17	5	102.3	14.92
	0.1	112.9, 108.7, 114.2, 110.9, 109.0	111.1	2.16	5		
Winter wheat (bread)	0.01	107.8, 99.1, 102.2, 104.2, 96.4	101.9	4.34	5	107.1	6.81
	0.1	102.6, 114.1, 111.3, 117.1, 116.1	112.2	5.17	5		
Winter wheat (germs)	0.01	110.0, 88.4, 101.3, 105.7, 101.1	101.3	7.98	5	98.4	6.35
	0.1	97.6, 95.7, 95.4, 95.5, 93.6	95.5	1.51	5		

Green beans (plants)	0.01	94.2, 111.2, 112.0, 119.0, 108.5	109.0	8.38	5	108.5	5.91
	0.1	103.7, 106.7, 109.6, 109.2, 111.2	108.1	2.71	5		
Green beans (pods)	0.01	93.2, 90.6, 91.9, 88.4, 88.3	90.5	2.38	5	95.7	6.15
	0.1	97.8, 102.8, 102.2, 102.5, 99.4	100.9	2.21	5		
Green beans (tips)	0.01	101.9, 96.7, 103.1, 98.4, 105.9	101.2	3.62	5	96.7	5.57
	0.1	92.1, 91.8, 93.6, 93.7, 90.2	92.3	1.54	5		
Green beans (cooked beans)	0.01	102.4, 105.4, 106.3, 107.6, 107.2	105.8	1.96	5	102.8	3.53
	0.1	97.5, 101.0, 101.7, 99.1, 99.5	99.8	1.68	5		
Rotational crop: Barley / green material	0.01	86.3, 96.1, 91.9, 90.3, 90.1	91.0	3.90	5	94.6	5.02
	0.1	98.0, 97.4, 99.9, 100.8, 94.5	98.1	2.50	5		
Rotational crop: Radish / roots	0.01	103.7, 101.7, 103.6, 105.8, 104.0	103.8	1.41	5	103.4	1.51
	0.1	100.6, 102.0, 104.3, 103.5, 104.8	103.0	1.67	5		
Rotational crop: Radish / leaves	0.01	109.5, 115.9, 111.7, 113.0, 104.5	110.9	3.84	5	108.1	3.92
	0.1	104.0, 107.5, 105.8, 104.0, 105.0	105.3	1.38	5		
Rotational crop: Lettuce / heads	0.01	114.0, 110.6, 111.7, 108.8, 109.5	110.9	1.84	5	110.6	2.98
	0.1	108.7, 112.5, 104.7, 116.7, 109.1	110.3	4.06	5		

* KCP 5.2/01b Report PBBZ-2011-07-DPL Supplement

Table A 100: Characteristics for the analytical method used for validation of azoxystrobin in different plant matrices

	Azoxystrobin
Specificity	<p>MS/MS determination was conducted by monitoring four mass transitions per analyte, $m/z = 372.2$ for quantification and $m/z = 344.2$, 329.1 and 172.1 for confirmation, however the validation was performed only for the quantification transition ($m/z 404 \rightarrow 372$) and the confirmation transition ($m/z 404 \rightarrow 329$).</p> <p>No interference at the retention time of Azoxystrobin above 30% of the LOQ and above the LOD were observed in the untreated control specimens of 30 matrices.</p> <p>In contrôle sample of summer barley yeast and hulls, some contamination at the retention time of Azoxystrobin were observed. However the area of interferinf peak not exceeded 30% of the LOQ area response.</p> <p>No interference at the retention time of Azoxystrobin-Z-isomer above 30% of the LOQ and above the LOD were observed in the untreated control specimens of all 32 matrices.</p> <p>The method is specific for Azoxystrobin and Azoxystrobin-Z-isomer.</p> <p>Representative chromatograms for the quantification transition ($m/z 404 \rightarrow 372$) and the confirmation transition ($m/z 404 \rightarrow 329$) and product ion spectra are provided.</p>
Calibration (type, number of data points)	<p>The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99.</p> <p>Please see table A101 below.</p>
Calibration range	<p>Linearity was confirmed over the calibration range 0.0001 – 1.00 $\mu\text{g/mL}$ (10.0 $\mu\text{g/mL}$ for green beans plants) ($n = 9$ or 10, corresponding to analyte concentrations of 0.003 mg/kg to 0.2 mg/kg (prothioconazole-desthio) in matrix samples.</p>
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	<p>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.</p> <p>The limit of detection (LOD) was set at the level of the lowest calibration standard concentration used with the signal three times greater than the background noise ($S/N \geq 3$).</p> <p>The LOD for azoxystrobin and azoxystrobin-Z-isomer was estimated at 0.001 – 0.004 mg/kg.</p>

	Azoxystrobin
Stability of standards and extracts	All samples were measured just after sample preparation (within 24 h). The stability of the Azoxystrobin and Azoxystrobin-Z-isomer in the final extract from untreated control matrix spiked with analytical standards at 0.1 µg/mL and stored at room and refrigerated temperature were checked.

Table A 101: Linearity of detector response

Analyte	Matrix	Transition	Linearity data
Azoxystrobin	Oilseed rape (grain)	404 → 372 m/z	y = 5688566 x - 4367 r = 1.0000
		404 → 329 m/z*	y = 1792757 x - 3121 r = 0.9999
	Oilseed rape (cake)	404 → 372 m/z	y = 5439246 x - 7913 r = 1.0000
		404 → 329 m/z*	y = 1762358 x - 3886 r = 0.9999
	Oilseed rape oil	404 → 372 m/z	y = 6459026 x - 11160 r = 1.0000
		404 → 329 m/z*	y = 2026740 x - 4511 r = 0.9998
	Summer barley grain	404 → 372 m/z	y = 1586121 x - 4449 r = 0.9999
		404 → 329 m/z*	y = 403724 x - 1581 r = 0.9996
	Summer barley straw	404 → 372 m/z	y = 10305718 x - 20494 r = 1.0000
		404 → 329 m/z*	y = 3439865 x - 4511 r = 0.9999
	Summer barley malt	404 → 372 m/z	y = 4939558 x - 14872 r = 0.9999
		404 → 329 m/z*	y = 1664055 x - 6825 r = 0.9997
	Summer barley wort	404 → 372 m/z	y = 2368753 x - 6332 r = 0.9999
		404 → 329 m/z*	y = 791602 x - 3266 r = 0.9997
	Summer barley germs	404 → 372 m/z	y = 9666344 x - 4482 r = 0.9999
		404 → 329 m/z*	y = 3283428 x - 5073 r = 0.9998
	Summer barley spent grain	404 → 372 m/z	y = 2109588 x - 8934 r = 0.9997
		404 → 329 m/z*	y = 682610 x - 3374 r = 0.9992
	Summer barley yeast	404 → 372 m/z	y = 2408160 x - 8402 r = 0.9999
		404 → 329 m/z*	y = 785767 x - 3506 r = 0.9996
	Summer barley waste	404 → 372 m/z	y = 2363142 x - 8835 r = 0.9998
		404 → 329 m/z*	y = 798924 x - 4722 r = 0.9993
	Summer barley beer	404 → 372 m/z	y = 2136985 x - 7767 r = 0.9999
		404 → 329 m/z*	y = 702343 x - 4068 r = 0.9996
	Summer barley pot barley	404 → 372 m/z	y = 1525239 x - 4719 r = 0.9999
		404 → 329 m/z*	y = 386746 x - 1443 r = 0.9998
	Summer barley hulls	404 → 372 m/z	y = 5274105 x - 21180 r = 0.9998
		404 → 329 m/z*	y = 1782576 x - 11835 r = 0.9993
	Winter wheat green material	404 → 372 m/z	y = 5756397 x - 24470 r = 0.9996
		404 → 329 m/z*	y = 1842793 x - 9038 r = 0.9992
	Winter wheat grain	404 → 372 m/z	y = 2320493 x - 7022 r = 0.9998
		404 → 329 m/z*	y = 576904 x - 2322 r = 0.9994
	Winter wheat straw	404 → 372 m/z	y = 4871575 x - 6685 r = 0.9999
		404 → 329 m/z*	y = 1642283 x - 7485 r = 0.9998
	Winter wheat water	404 → 372 m/z	y = 2091850 x - 6821 r = 0.9999
		404 → 329 m/z*	y = 682951 x - 2914 r = 0.9997
	Winter wheat bran	404 → 372 m/z	y = 1966206 x - 8131 r = 0.9997
		404 → 329 m/z*	y = 496629 x - 3608 r = 0.999
	Winter wheat middling	404 → 372 m/z	y = 1638858 x - 5429 r = 0.9999
		404 → 329 m/z*	y = 401012 x - 2247 r = 0.9996
	Winter wheat flour	404 → 372 m/z	y = 1977484 x - 7850 r = 0.9998

Analyte	Matrix	Transition	Linearity data
	Winter wheat dough	404 → 329 m/z*	y = 485154 x - 3236 r = 0.9991
		404 → 372 m/z	y = 2045780 x - 9277 r = 0.9997
	Winter wheat bread	404 → 329 m/z*	y = 670628 x - 4063 r = 0.9991
		404 → 372 m/z	y = 2052056 x - 8590 r = 0.9999
	Winter wheat germs	404 → 329 m/z*	y = 687200 x - 5102 r = 0.9994
		404 → 372 m/z	y = 9577415 x - 19402 r = 1.0000
	Green beans plants	404 → 329 m/z*	y = 32675149 x - 9912 r = 0.9999
		404 → 372 m/z	y = 5602672 x - 34370 r = 1.0000
	Green beans pods	404 → 329 m/z*	y = 1722876 x - 9921 r = 0.9999
		404 → 372 m/z	y = 16138921 x - 8864 r = 1.0000
	Green beans tips	404 → 329 m/z*	y = 4808082 x - 3406 r = 0.9999
		404 → 372 m/z	y = 6212054 x - 3783 r = 1.0000
	Green beans cooked	404 → 329 m/z*	y = 1899227 x - 3428 r = 0.9998
		404 → 372 m/z	y = 5389498 x - 5278 r = 1.0000
	Barley green material	404 → 329 m/z*	y = 1714881 x - 2900 r = 0.9999
		404 → 372 m/z	y = 8557118 x - 42667 r = 0.9993
	Radish roots	404 → 329 m/z*	y = 2897995 x - 20083 r = 0.998
		404 → 372 m/z	y = 6024128 x - 16097 r = 0.9999
	Radish leaves	404 → 329 m/z*	y = 2014295 x - 6814 r = 0.9998
		404 → 372 m/z	y = 8202506 x - 28789 r = 0.9998
	Lettuce heads	404 → 329 m/z*	y = 2786891 x - 17435 r = 0.9995
		404 → 372 m/z	y = 8066112 x - 19010 r = 1.0000
Azoxystrobin-Z-isomer	Oilseed rape (grain)	404 → 329 m/z*	y = 2699534 x - 9773 r = 0.9999
		404 → 372 m/z	y = 4087148 x - 2177, r = 1.0000
	Oilseed rape (cake)	404 → 329 m/z*	y = 1168112 x - 1707 r = 0.9999
		404 → 372 m/z	y = 3974879 x - 6735 r = 1.0000
	Oilseed rape oil	404 → 329 m/z*	y = 1141248 x - 2797 r = 0.9999
		404 → 372 m/z	y = 4570137 x - 6198 r = 1.0000
	Summer barley grain	404 → 329 m/z*	y = 1298281 x - 3194 r = 0.9999
		404 → 372 m/z	y = 1076424 x - 2718 r = 1.0000
	Summer barley straw	404 → 329 m/z*	y = 271616 x - 1017 r = 0.9996
		404 → 372 m/z	y = 7716770 x - 17969 r = 0.9999
	Summer barley malt	404 → 329 m/z*	y = 2388578 x - 7360 r = 0.9998
		404 → 372 m/z	y = 3543769 x - 10410 r = 0.9999
	Summer barley wort	404 → 329 m/z*	y = 1087055 x - 4363 r = 0.9998
		404 → 372 m/z	y = 1566838 x - 4931 r = 0.9999
	Summer barley germs	404 → 329 m/z*	y = 477611 x - 2093 r = 0.9998
		404 → 372 m/z	y = 7120545 x - 8656 r = 1.0000
	Summer barley spent grain	404 → 329 m/z*	y = 2199011 x - 4656 r = 0.9999
		404 → 372 m/z	y = 1384741 x - 6856 r = 0.9997
	Summer barley yeast	404 → 329 m/z*	y = 399701 x - 2118 r = 0.999
		404 → 372 m/z	y = 1590882 x - 5687 r = 0.9999
	Summer barley waste	404 → 329 m/z*	y = 475402 x - 2565 r = 0.9997
		404 → 372 m/z	y = 1576775 x - 7117 r = 0.9999
	Summer barley beer	404 → 329 m/z*	y = 478277 x - 2822 r = 0.9993
		404 → 372 m/z	y = 1387429 x - 5320 r = 0.9999
		404 → 329 m/z*	y = 415399 x - 1856 r = 0.9998
		404 → 372 m/z	

Analyte	Matrix	Transition	Linearity data
	Summer barley pot barley	404 → 372 m/z	y = 1001647 x - 2543 r = 1.0000
		404 → 329 m/z*	y = 253576 x - 877 r = 0.9998
	Summer barley hulls	404 → 372 m/z	y = 3347288 x - 13318 r = 0.9999
		404 → 329 m/z*	y = 1032489 x - 4951 r = 0.9995
	Winter wheat green material	404 → 372 m/z	y = 4156693 x - 16503 r = 0.9997
		404 → 329 m/z*	y = 1195725 x - 5570 r = 0.9992
	Winter wheat grain	404 → 372 m/z	y = 1642191 x - 5180 r = 0.9998
		404 → 329 m/z*	y = 408371 x - 1863 r = 0.9995
	Winter wheat straw	404 → 372 m/z	y = 3237343 x - 4608 r = 0.9999
		404 → 329 m/z*	y = 997201 x - 4125 r = 0.9999
	Winter wheat water	404 → 372 m/z	y = 1371299 x - 5187 r = 0.9999
		404 → 329 m/z*	y = 411605 x - 2425 r = 0.9997
	Winter wheat bran	404 → 372 m/z	y = 1263934 x - 5282 r = 0.9998
		404 → 329 m/z*	y = 324680 x - 2160 r = 0.999
	Winter wheat middling	404 → 372 m/z	y = 1073123 x - 4684 r = 0.9998
		404 → 329 m/z*	y = 264475 x - 1738 r = 0.9992
	Winter wheat flour	404 → 372 m/z	y = 1282566 x - 4384 r = 0.9998
		404 → 329 m/z*	y = 317664 x - 2039 r = 0.9991
	Winter wheat dough	404 → 372 m/z	y = 1316058 x - 6353 r = 0.9999
		404 → 329 m/z*	y = 389203 x - 2576 r = 0.9995
	Winter wheat bread	404 → 372 m/z	y = 1319798 x - 4802 r = 0.9999
		404 → 329 m/z*	y = 399509 x - 2794 r = 0.9995
	Winter wheat germs	404 → 372 m/z	y = 7043370 x - 17262 r = 0.9999
		404 → 329 m/z*	y = 2168426 x - 7840 r = 0.9998
	Green beans plants	404 → 372 m/z	y = 4063636 x - 27665 r = 1.0000
		404 → 329 m/z*	y = 1144352 x - 7207 r = 0.9999
	Green beans pods	404 → 372 m/z	y = 13991796 x - 2421 r = 1.0000
		404 → 329 m/z*	y = 3809640 x - 1195 r = 0.9999
	Green beans tips	404 → 372 m/z	y = 4715005 x - 1958 r = 1.0000
		404 → 329 m/z*	y = 1320175 x - 2208 r = 0.9998
	Green beans cooked	404 → 372 m/z	y = 3970419 x - 2039 r = 0.9999
		404 → 329 m/z*	y = 1146833 x - 1337 r = 0.9998
	Barley green material	404 → 372 m/z	y = 6151595 x - 31130 r = 0.9993
		404 → 329 m/z*	y = 1876569 x - 13279 r = 0.998
	Radish roots	404 → 372 m/z	y = 4319276 x - 10005 r = 1.0000
		404 → 329 m/z*	y = 1333611 x - 4880 r = 0.9998
	Radish leaves	404 → 372 m/z	y = 6087588 x - 19085 r = 0.9999
		404 → 329 m/z*	y = 1882415 x - 11105 r = 0.9995
	Lettuce heads	404 → 372 m/z	y = 5844829 x - 12446 r = 1.0000
		404 → 329 m/z*	y = 1791326 x - 7293 r = 0.9999

* KCP 5.2/01b Report PBBZ-2011-07-DPL Supplement

Conclusion

This analytical method for the determination of azoxystrobin and azoxystrobin-Z-isomer content in various plant matrices (high water content, high oil content and high protein/high starch content) has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for azoxystrobin and azoxystrobin-Z-isomer for each matrix

group.

A 2.1.2.1.1.2 Independent laboratory validation

Comments of zRMS:	<p>The method has been independently validated according to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99 rev. 4 for the determination of residues of azoxystrobin and its Z-isomer in winter wheat (whole plant and grain) and oilseed rape (grain) with the LOQ of 0.01 mg/kg.</p> <p>Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes and matrices at each level.</p> <p>The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met.</p> <p>The method is acceptable.</p>
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Reference:	KCP 5.2/02
Report	Azoxystrobin and its metabolite Z-isomer: Independent Laboratory Validation (ILV) of an analytical method for the determination of residues in winter wheat (whole plant and grain) and in oilseed rape (grain), Lefresne S., 2011, Report N°NUFARM/AZO/11.01
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of this study was to perform an independent laboratory validation of the method developed by the “Food Safety Laboratory (FSL)” for the determination of residues of azoxystrobin and its Z-isomer in winter wheat (whole plant and grain) and oilseed rape (grain).

Residues of Azoxystrobin and its Z-isomer were extracted from winter wheat (whole plant and grain) and oilseed rape (grain) by maceration with an acetonitrile/water mixture (for grain of oilseed rape, hexane is added). Magnesium sulfate, sodium chloride and buffering citrate salts were added (QuEChERS technique). For whole plant of wheat and grain of oilseed rape an aliquot was cleaned-up by dispersive solid phase extraction (d-SPE). The determination is performed by liquid chromatography with detection by mass spectrometric in tandem (LC-MS/MS). The limit of quantification (LOQ) is 0.01 mg/kg.

Only minor modifications were made to the original method of analysis:

- Samples were shaken vigorously manually instead by Vortex-type shaker,
- Centrifugation speeds were altered from 8500 rpm (4847 g) or 72000 rpm (5216 g) to 45000 rpm (3000 g) due to limitations on rotational speed of the equipment,
- The 100 μL of acetonitrile were not added just before the filtration of the final extract. The calibration curves were performed in a final volume of 1 mL instead of 1.1 mL.

No other modifications to the original method of analysis were made that were considered to be significant or to have any effect on the quality of the data.

Analytical conditions

Two analytical systems were used as part of this study.

The analytical system 1 was used for the determination of residues of azoxystrobin and its Z-isomer in winter wheat (whole plant and grain) at the LOQ and 10 xLOQ levels, and in oilseed rape (grain) at the LOQ level only.

The analytical system 2 was used only for the determination of residues of azoxystrobin and its Z-isomer in oilseed rape (grain) at the 10 x LOQ level.

Analytical system 1 LC condition

System: Varian 1200L

Autosampler Varian 410

Pump LC Varian ProStar 210

HiP – ALS G1367C

Workstation (Varian Star 6.42)

Column: C₁₈ Pyramid, 500 x 2.0mm, 5µm

Flow: 0.2 mL/min

Mobile phase

Eluent A: Ultra puer water/methanol/glacial acetic acid (98/5/0.05, v/v) filtered on Nylon® filter
0.20µm

Eluent B: Methanol/ glacial acetic acid (100/0.05, v/v)

Gradient:

Time (min)	% A	% B
0:00	100	0
10:00	40	60
15:00	40	60
15:06	0	100
17:00	0	100
17:06	100	0
21:00	100	0

Injection volume: 40 µL

Retention time:

Azoxystrobin: About 13.7 min

Azoxystrobin-Z-isomer: About 13.1 min

MS conditions

Detector: ALC1200

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Capillary voltage: 40 V

Temperature: 250°C

Scan type: MRM (Multiple Reaction Monitoring)

Mass transitions:

Azoxystrobin: m/z 404 → 372, m/z 404 → 344 and m/z 404 → 372 (total signal of the 3 transitions used for quantification)

Azoxystrobin-Z-isomer: m/z 404 → 372, m/z 404 → 344 and m/z 404 → 372 (total signal of the 3 transitions used for quantification)

Analytical system 2

LC condition

System: API4000

Autosampler, Pump LC (Ultimate 3000 RS)

Workstation (Varian Star 6.42)

Column: Kinetex C18, 100 x 2.1mm, 1.7µm

Column temperature: 40°C

Flow: 0.45 mL/min

Mobile phase

Eluent A: Water/glacial acetic acid (100/0.1% v/v) + 5mM ammonium acetate filtered on Nylon® filter 0.20µm

Eluent B: Methanol/ glacial acetic acid (100/0.1 % v/v) + 5mM ammonium acetate

Gradient:

Time (min)	% A	% B
0	50	50
1	50	50
2	30	70
3	30	70
4	0	100
5	0	100

6	50	50
8	50	50

Injection volume: 20 µL

Retention time:

Azoxystrobin: About 3.0-3.1 min

Azoxystrobin-Z-isomer: About 2.2-2.3 min

MS conditions

Detector: API4000

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Ionspray voltage: 5500 V

Temperature: 700°C

Scan type: MRM (Multiple Reaction Monitoring)

Mass transitions:

Azoxystrobin: m/z 404 → 372 (used for quantification), m/z 404 → 344 (used for qualification)

Azoxystrobin-Z-isomer: m/z 404 → 372 (used for quantification), m/z 404 → 344 (used for qualification)

Results and discussions

Table A 102: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Transition m/z 404 → 372 (Proposed for Quantification)							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Wheat (grain)	0.01 *	106, 108, 109, 98, 97, 95	102	6	6	103	5
	0.1 *	103, 99, 97, 111, 105	103	5	5		
Wheat (whole plant)	0.01 *	104, 90, 95, 82, 84	91	10	5	92	8
	0.1 *	93, 97, 83, 91, 98	92	6	5		
Oilseed rape (grain)	0.01 *	86, 81, 86, 77, 69	80	9	5	85	10
	0.1 **	94, 93, 82, 95, 90	91	6	5		

* results obtained with the analytical system 1

** results obtained with the analytical system 2 (quantification transition only)

Table A 103: Recovery results from method validation of azoxystrobin-Z-isomer using the analytical method

Azoxystrobin Z-isomer							
Transition m/z 404 → 372 (Proposed for Quantification)							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Wheat (grain)	0.01 *	109, 113, 110, 98, 97	105	8	5	105	6
	0.1 *	107, 99, 106, 100, 112	105	5	5		
Wheat (whole plant)	0.01 *	101, 100, 98, 78, 100	96	10	5	102	17
	0.1 *	115, 140, 81, 110, 96	108	20	5		
Oilseed rape (grain)	0.01 *	92, 86, 81, 103, 90	90	9	5	91	7
	0.1 **	96, 93, 83, 93, 94	92	6	5		

* results obtained with the analytical system 1

** results obtained with the analytical system 2 (quantification transition only)

Table A 104: Characteristics for the analytical method used for validation of azoxystrobin and azoxystrobin-Z-isomer in wheat and oilseed rape

	Azoxystrobin and Azoxystrobin-Z-isomer
Specificity	<p>The LC-MS/MS chromatographic method is highly specific for both analytical system 1 and 2.</p> <p>A reagent blank showed that no interference due to the reagents was detected (interference due to the substrate are less than 30% of the LOQ). For each specimen and each item, the specificity of the method has been demonstrated.</p> <p>Representative chromatogram for winter wheat (whole plant and grain) and analyte representing standards, samples, sample fortified at the LOQ and sample fortified at 10 x LOQ and showing reagent blank extracts are provided for the analytical system 1.</p> <p>Representative chromatogram for oilseed rape (grain) and analyte representing standards, samples, sample fortified at the LOQ and showing reagent blank extracts are provided for the analytical system 1.</p> <p>Representative chromatogram for oilseed rape (grain) and analyte representing standards, samples, sample fortified at the 10 x LOQ and showing reagent blank extracts are provided for the analytical system 2.</p>
Calibration (type, number of data points)	<p>The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99.</p> <p>Please see table A105 below.</p>
Calibration range	<p>For azoxystrobin, the linearity was confirmed over the calibration range 1.5 – 15 µg/L ($n = 6$) for whole plant of wheat (equivalent to 0.003 – 0.030 mg/kg), 1.5 – 20.0 µg/L ($n = 7$) for grain of wheat (equivalent to 0.003 – 0.040 mg/kg) and 0.5 – 5 µg/L ($n = 6$) for grain of oilseed grain (equivalent to 0.003 – 0.033 mg/kg).</p> <p>For z-azoxystrobin, the linearity was confirmed over the calibration range 3 – 15 µg/L ($n = 5$) for whole plant of wheat (equivalent to 0.006 – 0.030 mg/kg), 2.5 – 20.0 µg/L ($n = 6$) for grain of wheat (equivalent to 0.005 – 0.033 mg/kg) and 0.5 – 5 µg/L ($n = 6$) for grain of oilseed grain (equivalent to 0.003 – 0.033 mg/kg).</p>
Assessment of matrix effects is presented	No. Matrix matched calibration standards are used.
Limit of determination/quantification	<p>The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ of azoxystrobin and its Z-isomer is 0.010 mg/kg.</p> <p>The LOD is considered as the lowest calibration standard used:</p> <ul style="list-style-type: none"> - 0.003 mg/kg in wheat (whole plant and grain) and oilseed grain for azoxystrobin, - 0.003 mg/kg in oilseed grain for Z-azoxystrobin, - 0.005 mg/kg in wheat grain for Z-azoxystrobin, - 0.006 mg/kg in whole plant of wheat for Z-azoxystrobin.
Stability of standards and extracts	The final extracts fortified at 10 x LOQ level (0.1 mg/kg) were considered to be stable when stored 63h at $< -18^{\circ}\text{C}$ or 24h at $< -18^{\circ}\text{C}$ then 12 hours at 8°C then 15 minutes at room temperature.

Table A 105: Linearity of detector response

Azoxystrobin	
Matrix	Linearity data
Wheat (whole plant)	$y = 424680 x + 216872$, $r = 0.9987$ ($n = 6$)
Wheat (grain)	$y = 2 \times 10^6 x + 505963$, $r = 0.9992$ ($n = 7$)
Oilseed rape (grain)	$y = 2 \times 10^6 x + 647633$, $r = 0.9992$ ($n = 6$)

Azoxystrobin-Z-isomer	
Transition	Linearity data
Wheat (whole plant)	$y = 113228 x + 62143$, $r = 0.9996$ ($n = 5$)
Wheat (grain)	$y = 473477 x + 853941$, $r = 0.9958$ ($n = 6$)
Oilseed rape (grain)	$y = 1 \times 10^6 x + 352168$, $r = 0.9978$ ($n = 6$)

Conclusion

An independent laboratory validation of this analytical method has been performed on plant matrices (high water content, high oil content and high protein/high starch content). The method has been acceptably

validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled for an independent laboratory validation. The Limit of Quantification was 0.01 mg/kg for azoxystrobin and azoxystrobin-Z-isomer for each matrix groups.

A 2.1.2.1.1.3 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.1.1.4 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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.

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

A 2.1.2.2 Description of analytical methods for the determination of residues in animal origin

A 2.1.2.2.1 Prothioconazole residues in animal matrices

A 2.1.2.2.1.1 Method validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CF-3307/ (January 2023). This method has not been reassessed in the framework of this application.</p> <p><i>The analytical method 01009 was successfully validated for the determination of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxydesthio, and JAU 6476-4,5-dihydroxy-desthio in/on matrices of animal origin: milk, muscle, kidney, liver, fat and egg.</i></p> <p><i>Residues of all analytes were determined using HPLC-MS/MS.</i></p> <p><i>The Limit of Quantification (LOQ) for each analyte is 0.01 mg/kg (expressed as JAU 6476-desthio equivalents) in all matrices tested.</i></p> <p><i>Mean recoveries for all matrices per fortification level were between 70 and 103% for all mass transitions. The overall mean recoveries per matrix were between 75% and 101% with RSDs of up to 13.5% (n = 10). Relative standard deviations per analyte, fortification level, and matrix were below 20% (n = 5) for both transitions.</i></p> <p><i>All method validation data are in compliance with the guideline requirements for European enforcement methods (SANCO/825/00 rev. 8.1.).</i></p> <p><i>The method is acceptable.</i></p>
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Reference: KCP 5.2/03

Report Analytical method 01009 for the determination of residues of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxy-desthio, and JAU 6476-4,5-dihydroxy-desthio in/on matrices of animal origin by HPLC-MS/MS, Schulte, G.; Oel, D., 2014, Report No. M-279725-03-1

Guideline(s): SANCO/825/00 rev.7

Deviations: No

GLP: Yes

Acceptability: Yes

The objective of this study is to validate the analytical enforcement method 01009 for the determination of relevant residues of prothioconazole (Prothioconazole-desthio, Prothioconazole-3-hydroxy-desthio, Prothioconazole-4-hydroxy-desthio, Prothioconazole-3,4-dihydroxy-desthio, and Prothioconazole-4,5-dihydroxy-desthio) in/on matrices of animal origin by HPLC-MS/MS. The method was validated in the following matrices: cattle (milk, muscle, kidney, liver, fat) and poultry (egg).

Materials and methods

The analytical method 01009 was developed for the determination of relevant residues of prothioconazole (prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-3,4-dihydroxy-desthio, and prothioconazole-4,5-dihydroxy-desthio) in/on matrices of animal origin. Residues were extracted from cattle (milk, muscle, kidney, liver, fat) and poultry (egg) with acetonitrile / water (4/1, v/v) using a high-speed blender. Subsequently, the solutions were refluxed for 2 hours with 5 N HCl. This hydrolysis step cleaves conjugates to agylcones and converts the metabolites with diene structure back to aromatic compounds. Residues of all analytes were determined using HPLC-MS/MS. Residues were quantified against matrix-matched standards.

Analytical conditions

Analytical system: HPLC-MS/MS

Mass transitions:

prothioconazole -desthio: m/z 312→70 or 312→125

prothioconazole-3-OH-desthio: m/z 328→70 or 328→141

prothioconazole -4-OH-desthio: m/z 328→70 or 328→141

prothioconazole-3,4-di-OH-desthio: m/z 344→70 or 344→157

prothioconazole -4,5-di-OH-desthio: m/z 344→70 or 344→157)

Results and discussions

Accuracy:

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with the respective analytes at concentrations of 0.01 and 0.10 mg/kg. Results were within guideline requirements (mean recovery 70-120%; RSD \leq 20%).

For the 1st mass transition, overall mean recoveries for all matrices were in a range of 88-97% for JAU 6476-desthio, 91-99% for JAU 6476-3-hydroxy-desthio, 90-99% for JAU 6476-4-hydroxy-desthio, 76-95% for JAU 6476-3,4-dihydroxy-desthio and in the range of 85-97% for JAU 6476-4,5-dihydroxy-desthio. The results relevant to the precision and accuracy in eggs only are summarised in detail in the tables below.

Table A 106: Recovery results from method validation of prothioconazole residues using the analytical method

Analyte	Sample Material	Fortification Level [mg/kg]**	Data per fortification level			Overall	
			Recoveries [%] Individual values	Mean [%]	RSD* [%]	Mean [%]	RSD* [%]
JAU 6476-desthio quantitation <i>m/z</i> 312 \rightarrow 70	poultry (egg)	0.01	91, 90, 91, 94, 93	92	1.9	90	3.0
		0.10	86, 91, 88, 86, 88	88	2.3		
JAU 6476-desthio confirmation <i>m/z</i> 312 \rightarrow 125	poultry (egg)	0.01	93, 89, 87, 89, 84	88	3.9	88	3.0
		0.10	87, 91, 86, 88, 89	88	2.1		

Precision:

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels of 0.01 and 0.10 mg/kg for all matrices. All RSD values were well below 20%. For all analytes, the RSD of the repeatability tests at each recovery set ranged from 0.9 to 18.5% for the 1st MRM and from 1.3 to 18.0% for the second MRM.

Table A 107: Characteristics for the analytical method used for validation of Prothioconazole residues in animal matrices

	Prothioconazole residues
Specificity	The high selectivity of the method results from the HPLC separation in combination with the very selective MS/MS detection. Apparent residues in control samples were below 30% of the LOQ. Two MRM transitions for quantitation and confirmation were monitored for each analyte (JAU 6476-desthio: <i>m/z</i> 312 \rightarrow 70 or 312 \rightarrow 125; JAU 6476-3-OH-desthio: <i>m/z</i> 328 \rightarrow 70 or 328 \rightarrow 141; JAU 6476-4-OH-desthio: <i>m/z</i> 328 \rightarrow 70 or 328 \rightarrow 141; JAU 6476-3,4-di-OH-desthio: <i>m/z</i> 344 \rightarrow 70 or 344 \rightarrow 157; JAU 6476-4,5-di-OH-desthio: <i>m/z</i> 344 \rightarrow 70 or 344 \rightarrow 157) and in each matrix tested.
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 (regression model: $y = a \cdot x + b$) performing with 1/x weighing. The correlation coefficients, <i>r</i> obtained were > 0.99 . Typical calibration equations for Prothioconazole-desthio (1 st MRM) are presented in the following: Egg: $y = 14930 \cdot x + 44$; Correlation Coefficient=0.9996
Calibration range	The linearity was confirmed over the calibration range 0.25 – 10 μ g/L (n = 7)
Assessment of matrix effects is presented	Matrix-matched standards were used for quantification.
Limit of determination/quantification	The Limit of Quantification (LOQ) for each analyte is 0.01 mg/kg (expressed as prothioconazole-desthio equivalents) in all matrices tested. The Limit of Detection (LOD) was estimated to be at least 30% of the LOQ (corresponding to 0.003 mg/kg) for all matrices tested.
Stability of standards and extracts	Final extract solutions were stable for at least 48 h in a refrigerator at 4°C \pm 3°C. Secondary standard solutions to be used for preparation of matrix-matched standards were prepared immediately before use.

A 2.1.2.2.1.2 Independent laboratory validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CF-3307/ (January 2023). This method has not been reassessed in the framework of this application.</p> <p><u>Conclusion:</u> <i>The BCS Analytical Method No. 010091 for the determination of residues of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxy-desthio, and JAU 6476-4,5- dihydroxy-desthio in/on animal matrices, exemplified for bovine meat, cow's milk, and whole egg (limit of quantification LOQ 0.01 mg/kg per analyte, expressed as JAU 6476-desthio equivalents) has been independently validated.</i> <i>For all specimen matrices, for all analytes, for each fortification level, and for both MS/MS transitions monitored, the overall recoveries per matrix and analyte were in the range between 87% and 103%, and the relative standard deviations (RSD) were $\leq 6\%$.</i> <i>The limit of quantification for the LC/MS/MS method was established at 0.01 mg/kg per analyte (expressed as JAU 6476-desthio equivalents). It is concluded that Bayer CropScience Method 01009 fulfils the reproducibility requirements as defined in EC Guidance document on residue analytical methods (SANCO/825/00 rev. 8.1) and is, therefore, applicable as enforcement method.</i></p> <p><u>Remark:</u> <i>Residue analysis of bovine meat, cow's milk, and whole egg was performed according to BCS Method 01009 with minor modifications due to slightly different laboratory procedures. These modifications were necessary for adaptation of the method to the instrumentation used in the present study and do not query the quality of the original method. No major impact on the method was expected.</i></p>
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Reference: KCP 5.2/04

Report Independent laboratory validation of Bayer CropScience method No. 01009 for the determination of residues of JAU 6476-desthio, JAU 6476-3-hydroxy-desthio, JAU 6476-4-hydroxy-desthio, JAU 6476-3,4-dihydroxy-desthio, and JAU 6476-4,5- dihydroxy-desthio in/on Matrices of Animal Origin by HPLC-MS/MS., Bacher, R., 2006, M-279818-01-1

Guideline(s): SANCO/825/00, rev.7

Deviations: No

GLP: Yes

Acceptability: Yes

The purpose of this study was to independently validate analytical enforcement method 01009 for the determination of relevant residues of prothioconazole (prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-3,4-dihydroxy-desthio, and prothioconazole-4,5-dihydroxy-desthio) in/on matrices of animal origin by HPLC-MS/MS. Therefore, bovine meat, cow's milk, and whole egg samples were spiked with all analytes at LOQ and 10fold LOQ and processed according to residue analytical method 01009 with minor modifications in extraction procedure. These modifications were necessary for the adaptation of the method to the instrumentation of the performing laboratory and do not query the quality of the original method.

Materials and methods

Methods were applied as in KCP 5.2/03, Schulte, G.; Oel, D., 2014, Report No. M-279725-03-1. Minor modifications in extraction procedure were applied. These modifications were necessary for the adaptation of the method to the instrumentation of the performing laboratory and do not query the quality of the original method.

Analytical conditions

Analytical system: HPLC-MS/MS

Mass transitions:

Analyte	Transition mass	
	for quantification	for confirmation
prothioconazole-desthio	m/z 312 → 70	m/z 312 → 125
prothioconazole -3-hydroxy-desthio	m/z 328 → 70	m/z 328 → 141
prothioconazole -4-hydroxy-desthio	m/z 328 → 70	m/z 328 → 141
prothioconazole -3,4-dihydroxy-desthio	m/z 344 → 70	m/z 344 → 157
prothioconazole -4,5-dihydroxy-desthio	m/z 344 → 70	m/z 344 → 157

Results and discussions

Accuracy:

Mean recoveries for all metabolites (prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-3,4-dihydroxy-desthio, and prothioconazole-4,5-dihydroxy-desthio) in all matrices (bovine meat, milk and egg) at both fortification levels (LOQ and 10foldLOQ) were well within the 70–120% range. Results showed that the overall recoveries per matrix and analyte for both transitions were between 87 to 103%. The results relevant to the precision and accuracy in eggs only are summarised in detail in the tables below.

Table A 108: Recovery results from method validation of prothioconazole residues using the analytical method (mass transitions used for quantification)

Analyte	Crop, matrix	FL [mg/kg]	Recoveries [%] Individual values	Data per fortification level			Overall	
				Mean [%]	RSD [%]	n	Mean [%]	RSD [%]
JAU6476-desthio quantitation m/z 312 → 70	Egg	0.01	91, 90, 90, 89, 88	90	1	5	88	3
		0.10	89, 91, 87, 84, 84	87	4	5		

n: number of replicates, RSD: relative standard deviation

Table A 109: Recovery results from method validation of prothioconazole residues using the analytical method (mass transitions used for confirmation)

Analyte	Crop, matrix	FL [mg/kg]	Recoveries [%] Individual values	Data per fortification level			Overall	
				Mean [%]	RSD [%]	n	Mean [%]	RSD [%]
JAU6476-desthio confirmation m/z 312 → 125	Egg	0.01	92, 88, 89, 85, 89	89	3	5	87	3
		0.10	88, 89, 86, 84, 82	86	3	5		

n: number of replicates, RSD: relative standard deviation

Table A 110: Characteristics for the analytical method used for validation of prothioconazole residues in animal matrices

	Prothioconazole residues
Specificity	LC/MS/MS using the Multi Reaction Monitoring (MRM) mode allows to detect prothioconazole-desthio and its metabolites at concentrations of as low as 0.10 ng/mL with 50-μL injections, therefore providing sufficient sensitivity to determine and to confirm residues of the analytes in the final extracts. Only minor interfering signals in the duplicate blank control specimens were detected (<30% of LOQ), resulting in a limit of detection (LOD) of 0.002 mg/kg per analyte for all matrices investigated.
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 (regression model: $y = a \cdot x + b$) performing with 1/x weighing. The correlation coefficients R obtained were > 0.99. Egg: $y = 187000 \cdot x - 6250$; Correlation Coefficient=0.9986
Calibration range	The linearity was confirmed over the calibration range 0.10 – 10 ng/mL (n = 5)
Assessment of matrix effects is presented	Matrix-matched standards were used for quantification.

	Prothioconazole residues
Limit of determination/quantification	The limit of quantification was established and validated at 0.01 mg/kg per analyte, expressed as prothioconazole-desthio equivalents) in bovine meat, milk and poultry egg.
Stability of standards and extracts	All standard solutions were stored refrigerated in amber glass bottles when not in use. Stability of all standard solutions during the course of the study was demonstrated by consistent LC/MS/MS results.

Conclusion

The analytical enforcement method 01009 was successfully independently validated for the determination of prothioconazole-desthio, prothioconazole-3-hydroxy-desthio, prothioconazole-4-hydroxy-desthio, prothioconazole-3,4-dihydroxy-desthio, and prothioconazole-4,5-dihydroxy-desthio in/on animal matrices exemplified for bovine meat, cow's milk, and whole egg by LC-MS/MS. The method complies with all criteria according to SANTE/2020/12830, Rev 2 and is therefore suitable as an enforcement method for food of animal origin and animal tissues.

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. 4 23 February 2022, 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 2.1.2.2.2 Prothioconazole and metabolites in animal origin matrices

A 2.1.2.2.2.1 Method validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust 250 EC (January 2023). This method has not been reassessed in the framework of this application.</p> <p><u>Conclusion:</u> <i>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-hydroxy-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.</i> <i>For sugar solution the lowest level was validated for 6.65 mg/kg prothioconazole.</i></p> <p><i>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 ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 1.</i> <i>The method is acceptable.</i></p>
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Reference:	KCP 5.2/05
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).

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).

Fortification was done for recovery samples.

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

Injection volume: 40 µL

Pre-column: Phenomenex HPLC guard column with 4 mm C18 cartridge

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

Time (min)	% A	% B
0	80	20
2	80	20
5	10	90
6.5	10	90
7.5	80	20
8.5	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

Injection volume: 5 µL

Pre-column: Phenomenex HPLC guard column with 4 mm C18 cartridge

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

Time (min)	% A	% B
0	70	30
1	70	30
3.5	1	99
4.5	1	99
4.6	70	30
6.0	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: Phenomenex UHPLC guard column with 2.1 mm C18 cartridge

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

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

Mobile phase B: Methanol + 5 mM Ammonium formate + 0.1% v/v formic acid

Flow: 0.8 mL/min

Column temperature: 60°C

Injection volume: 20 µL

Time (min)	% 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

Same for pollen method validation except:

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

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

Flow: 0.8 mL/min

Column temperature: 60°C

Injection volume: 25 µL

Time (min)	% A	% B
0	70	30
0.10	70	30
0.11	50	50
3.0	10	90
4.0	10	90
4.01	70	30
5.5	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

Pre-column: Phenomenex HPLC guard column with 4 mm Fusion RP cartridge

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

Injection volume: 40 µL

Time (min)	% A	% B
0	99	1
0.2	99	1
5	10	90
7	10	90
7.01	99	1
8.5	99	1

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

Pre-column: Phenomenex HPLC guard column with 4 mm C18 cartridge

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

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

Mobile phase B: Methanol + 0.5% v/v formic acid

Column temperature: 30°C

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

Time (min)	% A	% B	Flow (µL/min)
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0	95	5	525
5	5	95	525
6	5	95	700
6.01	95	5	700
7.5	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 111 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	312→70 <i>m/z</i> (Confirmation)	
		0.1	101	1				
		0.01	100	1	100	1		328→70 <i>m/z</i> (Quantification)
		0.1	100	1				
Prothioconazole- alpha-hydroxy- Desthio*	Pollen	0.01	109	4	107	4	328→70 <i>m/z</i> (Quantification)	
		0.1	105	2				
	Nectar	0.01	95	2	97	2		328→141 <i>m/z</i> (Confirmation)
		0.1	98	1				
	Honey	0.01	103	3	101	3	328→141 <i>m/z</i> (Confirmation)	
		0.1	100	2				
		0.01	105	4	103	4		328→141 <i>m/z</i> (Confirmation)
		0.1	101	2				
Prothioconazole-3- hydroxy-Desthio*	Pollen	0.01	100	7	102	5	328→70 <i>m/z</i> (Quantification)	
		0.1	103	2				
	Nectar	0.01	100	1	99	1		328→141 <i>m/z</i> (Confirmation)
		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→141 <i>m/z</i> (Confirmation)
		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				
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	70→43 <i>m/z</i> (Quantification) Hypercarb column	
		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				
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)
		0.1	111	2				
	Honey	0.01	111	4	106	6	157→88 <i>m/z</i> (Confirmation)	
		0.1	102	3				
0.01	113	2	109	5	157→88 <i>m/z</i> (Confirmation)			
0.1	104	5						

							Hypercarb column
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	
		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 Acid	Pollen	0.01	110	4	108	4	158→70 <i>m/z</i> (Quantification) Biphenyl column
		0.1	105	4			
	Nectar	0.01	103	3	104	2	158→70 <i>m/z</i> (Quantification) Hypercarb column
		0.1	104	1			
	Honey	0.01	96	9	97	6	
		0.1	97	1			
		0.01	105	8	102	7	158→43 <i>m/z</i> (Confirmation) Hypercarb column
		0.1	98	2			

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

Table A 112: 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 standards. Linear calibration functions were calculated by regression analysis. The correlation coefficients R obtained were > 0.99. Please refer to table A108 below.		
Calibration range	Please refer to table A113 below.		

	Group 1	Group 2	Group 3
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	<p>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).</p> <p>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.</p> <p>Lowest validated fortification level for sugar solution was 6.65 mg/kg and evaluated for one mass transition.</p>		
Stability of standards and extracts	<p>Stock solutions:</p> <p>Prothioconazole in acetonitrile stable for at least 41 days at 1 °C to 10 °C.</p> <p>Prothioconazole-desthio stable in acetonitrile for at least 41 days when at 1 °C to 10 °C.</p> <p>PTZ-alpha-hydroxy-desthio stable in acetonitrile for at least 120 at 1 °C to 10 °C.</p> <p>PTZ-3-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C.</p> <p>PTZ-4-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C.</p> <p>PTZ-5-hydroxy-desthio stable in acetonitrile for at least 120 days at 1 °C to 10 °C.</p> <p>PTZ-6-hydroxy-desthio stable in acetonitrile for at least 70 days at 1 °C to 10 °C.</p> <p>All analytes of group 3 stable in HPLC water for at least 76 at 1 °C to 10 °C.</p> <p>Extracts:</p> <p>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 113: Linearity of detector response

Analyte	Matrix/Transition	Calibration range	Equation	r
Prothioconazole	Pollen 344→154 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (Quantification)	0.075 – 7.5 ng/mL (0.003 – 0.3 mg/kg) n = 6	$y = 3.31e+005 \ x - 147$	0.9999
	Honey 328→141 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (Quantification)	0.09 – 7.5 ng/mL (0.003 – 0.25 mg/kg) n = 7	$y = 5.85e+003 \ x + 341$	0.9987

Analyte	Matrix/Transition	Calibration range	Equation	r
	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.2 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 2.1.2.2.3 Independent laboratory validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CA3301/Joust 250 EC (January 2023). This method has not been reassessed in the framework of this application.</p> <p><u>Conclusion:</u></p> <p><i>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.</i></p> <p><i>An LOQ of 0.01 mg/kg was confirmed for all analytes in honey.</i></p> <p><i>The method is acceptable.</i></p>
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Reference: KCP 5.2/06

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.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Methods were applied as in KCP 5.2/05 Kalathoor R., 2021, Report No. S20-09747. 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.2.

Results and discussions

Table A 114: 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) Hypercarb column
		0.1	101	5.0			
Triazole Alanine	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			
Triazole Acetic Acid	Honey	0.01	93.3	2.3	94.8	2.9	128→70 m/z (Quantification) Hypercarb column
		0.1	96.4	2.7			

Triazole Lactic Acid	Honey	0.01	95.3	3.9	94.3	9.1	128→70 m/z (Confirmation) Biphenyl column
		0.1	93.2	13			
		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 115: 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. 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 A116 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 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.</p> <p>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.</p>		

Table A 116: Linearity of detector response

Analyte	Matrix/Transition	Calibration range	Equation	r
Prothioconazole-desthio	Honey 312→125 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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 <i>m/z</i> (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.2 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.2.

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

A 2.1.2.2.3.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.2.3.2 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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 2.1.2.2.3.3 Azoxystrobin and metabolite in food stuff of animal origin (bovine's milk, poultry's eggs, bovine fat, muscle meat and liver)

2.1.2.2.3.3.1. Method validation

Comments of zRMS:	<p>The multi-residue method QuEChERS has been successfully validated according to the guidance documents SANCO/825/00, rev. 8.1 for the determination of residues of azoxystrobin and R230310 content in various animal origin matrices with the LOQ of 0.01 mg/kg. A highly specific detection system was used (MS/MS).</p> <p>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 acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met.</p> <p>The method is acceptable.</p>
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Reference:	KCP 5.2/07
Report	Laboratory validation of a method for the determination of Azoxystrobin and R230310 in Different matrices of animal origin, Siekmann D., 2017, Report N°S17-01577
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Azoxystrobin and R230310 were determined in different matrices of animal origin (bovine whole milk, poultry's eggs, bovine meat, bovine liver and bovine fat) using a liquid chromatography technique (HPLC-MS/MS, 2 mass transitions monitored) after addition of water and extraction with acetonitrile using QuEChERS method. After extraction, extracts were cleaned by adding primary secondary amine (PSA) and C18 material. Before injection, samples were diluted in water containing 0.05% of acetic acid.

Matrix-matched standards were used for quantification. The intended limit of quantification (LOQ) was 0.01 mg/kg in all matrix types for Azoxystrobin and R230310.

Analytical conditions

LC conditions

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

Pre-column: HPLC guard column with 4mm C18 cartridge

Column: MZ-Analysentechnik Kromasil 100-5 C18, 50mm x 3.0mm, 5µm

Column temperature: 40°C

Flow: 1 mL/min

Mobile phase

Eluent A: water with 0.2% v/v acetic acid

Eluent B: acetonitrile

Gradient:

Time (min)	% A	% B
0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10
5.0	90	10

Divert valve:

0.0 min to 1.0 min to waste

1.0 min to 3.5 min to MS

3.5 min to 5.1 min to waste

Injection volume: 20 µL

Retention time:

Azoxystrobin: About 2.3 min

R230310: About 2.0 min

MS conditions

System: SCIEX API 5500

Ionisation type: Electrospray ionisation (ESI, Turbolon Spray)

Polarity: Positive ion mode

Scan type: MS/MSn Multiple reaction monitoring (MRM)

Ionspray turbo heater: 400°C

Capillary voltage (IS) 5500 V

Azoxystrobin and R230310: 404 → 372 m/z proposed for quantification and 404 → 344 m/z proposed for confirmation

Results and discussions

Table A 117: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Bovine whole milk	0.01	105, 100, 101, 100, 109	103	4	5	101	4
	0.1	102, 97, 93, 99, 101	98	4	5		
Poultry's eggs	0.01	101, 103, 103, 103, 100	102	1	5	100	4
	0.1	104, 97, 94, 94, 96	97	4	5		
Bovine fat	0.01	103, 101, 96, 100, 97	99	3	5	98	3
	0.1	95, 94, 97, 98, 97	96	2	5		
Bovine muscle meat	0.01	89, 95, 100, 100, 97	96	5	5	96	3
	0.1	94, 92, 86, 92, 92	91	3	5		

Bovine liver	0.01	107, 105, 104, 96, 101	103	4	5	104	3
	0.1	103, 103, 104, 108, 104	104	2	5		
Transition m/z 404 \rightarrow 344 m/z (Proposed for Confirmation)							
Bovine whole milk	0.01	105, 100, 101, 98, 110	103	5	5	101	5
	0.1	102, 97, 93, 99, 100	98	3	5		
Poultry's eggs	0.01	101, 103, 103, 103, 100	102	1	5	100	4
	0.1	105, 98, 98, 94, 95	97	5	5		
Bovine fat	0.01	103, 101, 96, 100, 97	99	3	5	98	3
	0.1	95, 95, 97, 98, 97	96	1	5		
Bovine muscle meat	0.01	91, 97, 101, 100, 98	97	4	5	96	3
	0.1	93, 92, 86, 91, 92	91	3	5		
Bovine liver	0.01	108, 107, 104, 97, 102	104	4	5	104	3
	0.1	102, 103, 103, 107, 104	104	2	5		

Recoveries are without any blank correction

Table A 118: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)							
Bovine whole milk	0.01	104, 97, 103, 103, 112	104	5	5	102	5
	0.1	102, 96, 95, 102, 101	99	3	5		
Poultry's eggs	0.01	99, 99, 102, 102, 99	100	2	5	99	4
	0.1	104, 100, 94, 94, 95	97	5	5		
Bovine fat	0.01	102, 100, 97, 99, 97	99	2	5	98	2
	0.1	97, 95, 98, 98, 97	97	1	5		
Bovine muscle meat	0.01	90, 94, 99, 96, 97	95	4	5	96	3
	0.1	95, 91, 87, 91, 91	91	3	5		
Bovine liver	0.01	107, 102, 101, 96, 101	101	4	5	102	3
	0.1	103, 103, 101, 102, 103	102	1	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Confirmation)							
Bovine whole milk	0.01	103, 96, 103, 102, 112	103	6	5	101	5
	0.1	102, 97, 95, 101, 100	99	3	5		
Poultry's eggs	0.01	100,100, 102, 101, 99	100	1	5	99	3
	0.1	104, 101, 94, 94, 96	98	5	5		
Bovine fat	0.01	101, 99, 97, 99, 98	99	2	5	98	2
	0.1	97, 95, 98, 98, 97	97	1	5		
Bovine muscle meat	0.01	91, 95, 99, 97, 98	96	3	5	96	3
	0.1	96, 91, 87, 92, 92	92	4	5		
Bovine liver	0.01	108, 104, 101, 96, 102	102	4	5	103	3
	0.1	103, 103, 102, 103, 103	103	0	5		

Recoveries are without any blank correction

Table A 119: Characteristics for the analytical method used for validation of azoxystrobin and R230310 residues in animal origin matrices

	Azoxystrobin and R230310
Specificity	MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). Two control samples per matrix and analyte were extracted and analysed according to the method. For both mass transition, the samples showed no significant interference (above 30% of LOQ) at the retention time of the analytes in any investigated matrix. The method is highly specific. Representative chromatograms for each matrix and analyte representing control samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank extracts are provided (together with product ion spectra).
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 .

	Azoxystrobin and R230310
	Please see table A120 below.
Calibration range	Linearity was confirmed over the calibration range 0.1 – 10.0 ng/mL (n = 7), corresponding to a range from 0.002 mg/kg to 0.2 mg/kg in sample extract which corresponds to more than 30% of the LOQ to 200 % above the highest level of recovery validated.
Assessment of matrix effects is presented	Yes
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) is set at 30% of the LOQ which is 0.003 mg/kg.
Stability of standards and extracts	Stock solutions prepared in acetonitrile are considered to be stable when stored for 8 days at 1°C to 10°C in the dark. Calibration solutions prepared in blank matrix of bovine whole milk, poultry's eggs and bovine muscle meat are considered to be stable when stored for 8 days at 1°C to 10°C in the dark. Calibration solutions prepared in blank matrix of bovine liver and bovine fat are considered to be stable when stored for 9 days at 1°C to 10°C in the dark. Extracts of bovine whole milk, poultry's eggs, bovine muscle meat and bovine liver are considered to be stable when stored at 1 °C to 10 °C for 7 days in the dark. Extracts of bovine fat are considered to be stable when stored at 1 °C to 10 °C for 8 days in the dark.

Table A 120: Linearity of detector response

Azoxystrobin		
Matrix	Transition	Linearity data
Bovine whole milk	404 → 372 m/z (Quantification)	$y = 3.81 \times 10^5 x + 3.36 \times 10^3$, $r = 0.9999$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.12 \times 10^6 x + 1.03 \times 10^4$, $r = 0.9999$ (n = 7)
Poultry's eggs	404 → 372 m/z (Quantification)	$y = 4.06 \times 10^5 x + 2.79 \times 10^3$, $r = 1.0000$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.19 \times 10^6 x + 6.36 \times 10^3$, $r = 0.9999$ (n = 7)
Bovine fat	404 → 372 m/z (Quantification)	$y = 3.91 \times 10^5 x + 2.37 \times 10^3$, $r = 1.0000$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.14 \times 10^6 x + 7.21 \times 10^3$, $r = 1.0000$ (n = 7)
Bovine muscle meat	404 → 372 m/z (Quantification)	$y = 4.03 \times 10^5 x + 2.95 \times 10^3$, $r = 0.9998$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.17 \times 10^6 x + 1.13 \times 10^4$, $r = 0.9998$ (n = 7)
Bovine liver	404 → 372 m/z (Quantification)	$y = 4.31 \times 10^5 x + 824$, $r = 0.9998$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.24 \times 10^6 x + 8.89 \times 10^3$, $r = 0.9999$ (n = 7)

R230310		
Matrix	Transition	Linearity data
Bovine whole milk	404 → 372 m/z (Quantification)	$y = 3.06 \times 10^5 x + 4.62 \times 10^3$, $r = 0.9997$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 8.81 \times 10^5 x + 1.04 \times 10^4$, $r = 0.9998$ (n = 7)
Poultry's eggs	404 → 372 m/z (Quantification)	$y = 3.24 \times 10^5 x + 5.40 \times 10^3$, $r = 0.9996$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 9.41 \times 10^5 x + 1.52 \times 10^4$, $r = 0.9996$ (n = 7)
Bovine fat	404 → 372 m/z (Quantification)	$y = 3.22 \times 10^5 x + 1.53 \times 10^3$, $r = 1.0000$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 9.02 \times 10^5 x + 4.72 \times 10^3$, $r = 1.0000$ (n = 7)
Bovine muscle meat	404 → 372 m/z (Quantification)	$y = 2.99 \times 10^5 x + 3.21 \times 10^3$, $r = 0.9999$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 8.54 \times 10^5 x + 9.62 \times 10^3$, $r = 0.9999$ (n = 7)
Bovine liver	404 → 372 m/z (Quantification)	$y = 3.55 \times 10^5 x + 3.34 \times 10^3$, $r = 0.9999$ (n = 7)
	404 → 344 m/z (Confirmation)	$y = 1.00 \times 10^6 x + 1.05 \times 10^4$, $r = 0.9999$ (n = 7)

Conclusion

This analytical method for the determination of azoxystrobin and R230310 content in various animal origin matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements

were also fulfilled. The Limit of Quantification was 0.01 mg/kg for azoxystrobin and R230310.

2.1.2.2.3.3.2. Independent laboratory validation

Comments of zRMS:	The analytical method has been independently validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin and R230310 content in different animal origin matrices (bovine fat, muscle meat and whole milk). An LOQ of 0.01 mg/kg was confirmed for all azoxystrobin and R230310 in fat, meat and milk. The primary method is identical for all animal matrices, it is sufficient to perform the ILV with at least two of these matrices. The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met. The method is acceptable.
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Reference:	KCP 5.2/08
Report	Independent Laboratory validation of method for the determination of Azoxystrobin and R230310 in Different matrices of animal origin, Meyer M., 2017, Report N°S17-02332
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to independently validate an analytical method as described in study number S17-01577 for the determination of azoxystrobin and R230310 in different matrices of animal origin.

Azoxystrobin and R230310 were determined in different matrices of animal origin (bovine whole milk and fat, muscle meat) using a liquid chromatography technique (HPLC-MS/MS, 2 mass transitions monitored) after extraction with acetonitrile and cleaning with QuEChERS method. Before injection, samples were diluted in water containing 0.05% of acetic acid.

Matrix-matched calibration standards were used for quantification. The intended limit of quantification (LOQ) was 0.01 mg/kg in all matrix types for azoxystrobin and R230310.

Analytical conditions

LC conditions

System:

Pump + autosampler: Shimadzu LC20ADXR or LC-30AD Nexera X2, Shimadzu

Oven: CTO-20AC, Shimadzu

Detector: API 5500, API 6500 (Sciex)

Data acquisition: Analyst 1.5.1, Sciex or Analyst 1.6.2, Sciex

Pre-column: Phenomenex C18 cartridge

Column: Kromasil C18, 50mm x 3.0mm, 5µm

Column temperature: 40°C

Flow: 1 mL/min

Mobile phase

Eluent A: water with 0.2 % (v/v) acetic acid

Eluent B: acetonitrile

Gradient:

Time (min)	% A	% B
0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10

5.0	90	10
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Divert valve:

0.0 min to 1.0 min to waste

1.0 min to 3.5 min to MS

3.5 min to 5.1 min to waste

Injection volume: 25 µL

Retention time:

Azoxystrobin: About 2.3 min

R230310: About 2.0 min

MS conditions

Ionisation type: Electrospray ionisation (ESI, Turbolon Spray)

Polarity: Positive ion mode

Scan type: MS/MSn Multiple reaction monitoring (MRM)

Azoxystrobin and R230310: 404 → 372 m/z proposed for quantification and 404 → 344 m/z proposed for confirmation

Results and discussions

Table A 121: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Bovine fat	0.01	100, 95, 97, 95, 87	85	5	5	83	16
	0.1	62*, 76, 74, 73, 75	72	8	5		
Bovine muscle meat	0.01	62*, 98, 99, 98, 102	92	18	5	82	18
	0.1	73, 70, 72, 74, 75	73	3	5		
Bovine whole milk	0.01	102, 97, 98, 96, 99	98	2	5	86	15
	0.1	74, 74, 73, 72, 74	73	1	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Bovine fat	0.01	93, 97, 93, 91, 90	93	3	5	83	14
	0.1	63*, 76, 75, 72, 75	72	7	5		
Bovine muscle meat	0.01	55**, 96, 99, 99, 96	98	2	4	83	17
	0.1	73, 69*, 70, 72, 73	71	3	5		
Bovine whole milk	0.01	100, 107, 95, 106, 107	103	5	5	89	18
	0.1	74, 74, 75, 72, 76	74	2	5		

*This recovery is out of range of 70 – 110%. However, as the mean of recoveries at this concentration for this batch is within 70 – 110% and the mean of all recoveries for this batch is within 70 – 110%, therefore this batch is validated.

**Dixon test was applied to exclude one of the five initial values

Table A 122: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)							
Bovine fat	0.01	99, 99, 102, 98, 95	99	3	5	87	15
	0.1	69*, 78, 77, 75, 77	75	5	5		
Bovine muscle meat	0.01	75, 101, 99, 99, 103	95	12	5	85	16
	0.1	75, 71, 73, 73, 76	74	3	5		
Bovine whole milk	0.01	101, 100, 97, 94, 98	98	3	5	86	15
	0.1	73, 74, 74, 72, 74	73	1	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Confirmation)							
Bovine fat	0.01	103, 100, 102, 101, 105	102	2	5	87	18

	0.1	68*, 75, 74, 74, 72	73	4	5		
Bovine muscle meat	0.01	61*, 93, 84, 96, 87	84	16	5	79	13
	0.1	75, 73, 73, 74, 76	74	2	5		
Bovine whole milk	0.01	106, 104, 96, 95, 94	99	6	5	87	15
	0.1	74, 74, 76, 73, 77	75	2	5		

*This recovery is out of range of 70 – 110%. However, as the mean of recoveries at this concentration for this batch is within 70 – 110% and the mean of all recoveries for this batch is within 70 – 110%, therefore this batch is validated.

Table A 123: Characteristics for the analytical method used for validation of azoxystrobin and R230310 residues in animal origin matrices

	Azoxystrobin and R230310
Specificity	MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). A reagent blank and two control samples per matrix were extracted and analysed according to the method. For both mass transition, the samples showed no significant interference (above 30% of LOQ) at the retention time of the analytes in any investigated matrix. The method is highly specific. Representative chromatograms for each matrix and analyte representing control samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank extracts are provided (together with product ion spectra).
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A124 below.
Calibration range	Linearity was confirmed over the calibration range 0.1 – 10.0 ng/mL ($n = 6$), corresponding to a range from 0.002 mg/kg to 0.2 mg/kg in sample extract which corresponds to more than 30% of the LOQ to 200 % above the highest level of recovery validated.
Assessment of matrix effects is presented	Yes
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) is set at 30% of the LOQ which is 0.003 mg/kg.
Stability of standards and extracts	NA

Table A 124: Linearity of detector response

Azoxystrobin		
Matrix	Transition	Linearity data
Bovine whole milk	404 → 372 m/z (Quantification)	$y = 232340 x + 8860, r = 0.9993 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 46616 x + 1795, r = 0.9996 (n = 7)$
Bovine fat	404 → 372 m/z (Quantification)	$y = 247762 x + 10265, r = 0.9992 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 49948 x + 1849, r = 0.9997 (n = 7)$
Bovine muscle meat	404 → 372 m/z (Quantification)	$y = 238767 x + 7686, r = 0.9995 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 48755 x + 1245, r = 0.9996 (n = 7)$

R230310		
Matrix	Transition	Linearity data
Bovine whole milk	404 → 372 m/z (Quantification)	$y = 425905 x + 20292, r = 0.9985 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 29130 x + 870, r = 0.9989 (n = 7)$
Bovine fat	404 → 372 m/z (Quantification)	$y = 446640 x + 18282, r = 0.9993 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 31803 x + 615, r = 0.9993 (n = 7)$
Bovine muscle meat	404 → 372 m/z (Quantification)	$y = 434869 x + 17003, r = 0.9993 (n = 7)$
	404 → 344 m/z (Confirmation)	$y = 29434 x + 2336, r = 0.9998 (n = 7)$

Conclusion

This analytical method for the determination of azoxystrobin and R230310 content in various animal origin

matrices has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for azoxystrobin and R230310.

A 2.1.2.2.3.4 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.2.3.5 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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.

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

A 2.1.2.2.3.6 Azoxystrobin and metabolite in food stuff of animal origin (honey)

2.1.2.2.3.6.1. Method validation

Comments of zRMS:	The multi-residue method QuEChERS has been successfully validated according to the guidance documents SANTE/2020/12830 rev.2 for the determination of residues of azoxystrobin and R230310 content in honey with the LOQ of 0.01 mg/kg. Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$ for all analytes at each level. The method is acceptable.
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Reference: KCP 5.2/09

Report Azoxystrobin – Azoxystrobin (ICI5504): Validation of Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin and its Metabolite R230310 in Honey Matrices by LC-MS/MS, Harper H., 2022, Report No. 8485926

Guideline(s): SANTE/2020/012830 rev1

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and method

The objective of the study was to validate an analytical residue QuEChERS method for the determination of azoxystrobin and its metabolite R230310 in honey.

2 g of matrix (multiflower honey – characterisation information is presented in the study report) were transferred into a 50 mL polypropylene tube. 10 mL of water and 10 mL of acetonitrile were added and the tube was vigorously shaken for approximately 1 minute. The contents of a citrate tube was added and the tube was vigorously shaken for approximately 1 minute. The tube was centrifuged at 3500 rpm for 5 minutes. 1 mL of the upper acetonitrile phase was transferred into a QuEChERS Dispersive tube and was shaken vigorously by hand for approximately 1 minute. The sample was then centrifuged at 3500 rpm for 5 minutes. 400 μ L of the upper acetonitrile layer was transferred into a 15 mL polypropylene tube and completed up to 2 mL with water. The final extract was analysed for azoxystrobin and its metabolites R230310 using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit

of quantification (LOQ) for azoxystrobin and its metabolite R230310 was 0.01 mg/kg.

Analytical conditions

LC conditions

System: Waters Acquity UPLC System

Column: Acquity UPLC BEH C₁₈, 50mm x 2.1mm, 1.7µm

Column temperature: 40°C

Injection volume: 10 µL

Flow: 0.5 mL/min

Mobile phase

Eluent A: 0.2% acetic acid in water

Eluent B: Acetonitrile

Gradient:

Time (min)	% A	% B
0.0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10
5.0	90	10

Retention time:

Azoxystrobin: About 1.8 min

R230310: About 1.6 min

MS conditions

MS system : API 4000 Mass spectrometer, Applied Biosystems

Ionisation type: Ionspray

Polarity: Positive ion mode

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

Azoxystrobin and R230310: 404 → 372 m/z used for quantification and 404 → 344 m/z used for confirmation.

Results and discussions

Table A 125: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (used for Quantification)							
Honey	0.01	112, 120, 120, 123, 124	120	3.9	5	107	14.4
	0.1	77, 101, 99, 102, 93	94	10.9	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (used for Confirmation)							
Honey	0.01	115, 114, 115, 125, 125	119	4.8	5	107	14.1
	0.1	78, 104, 98, 98, 93	94	10.5	5		

Table A 126: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (used for Quantification)							
Honey	0.01	107, 127, 110, 113, 107	113	7.4	5	106	9.0
	0.1	90, 105, 103, 103, 99	100	6.0	5		

Transition m/z 404 → 344 m/z (used for Confirmation)							
Honey	0.01	105, 125, 103, 113, 108	111	7.9	5	107	7.5
	0.1	95, 105, 106, 106, 100	102	4.7	5		

Table A 127: Characteristics for the analytical method used for validation of azoxystrobin and R230310 in honey

	Azoxystrobin	R230310
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). The method is considered highly specific. No residues of azoxystrobin were detected above 30% of the LOQ in any of the control and reagent blank samples, indicating that no interferences were present at the retention time of azoxystrobin in the honey. Representative chromatograms representing control and blank samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent are provided. Azoxystrobin mass spectrum is also provided.	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). The method is considered highly specific. No residues of R230310 were detected above 30% of the LOQ in any of the control and reagent blank samples, indicating that no interferences were present at the retention time of R230310 in the honey. Representative chromatograms representing control and blank samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent are provided. Azoxystrobin mass spectrum is also 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 performed with 1/x weighing (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A128 below.	The linearity of the method was demonstrated using solvent calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x weighing (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A128 below.
Calibration range	Linearity was confirmed over the calibration range 0.1 – 5.0 ng/mL ($n = 9$), corresponding to a range from 0.0025 – 0.125 mg/kg in matrix which corresponds to 25% of the LOQ to at least 20 % above the highest analyte concentration in the final sample extracts.	Linearity was confirmed over the calibration range 0.1 – 5.0 ng/mL ($n = 9$), corresponding to a range from 0.0025 – 0.125 mg/kg in matrix which corresponds to 25% of the LOQ to at least 20 % above the highest analyte concentration in the final sample extracts.
Assessment of matrix effects is presented	Yes (insignificant)	Yes (insignificant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for azoxystrobin in honey. The LOD is considered as the lowest calibration standard used, 0.1 ng/mL corresponding to 0.0025 mg/kg (25% of the LOQ).	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for R230310 in honey. The LOD is considered as the lowest calibration standard used, 0.1 ng/mL corresponding to 0.0025 mg/kg (25% of the LOQ).
Stability of standards and extracts	The residues in final extracts (10 x LOQ) are considered to be stable when stored for 7 days between 2 and 8°C. Standard solutions prepared in acetonitrile/water (50/50 v/v) are considered to be stable when stored 208 days between 2 and 8°C.	The residues in final extracts (10 x LOQ) are considered to be stable when stored for 7 days between 2 and 8°C. Standard solutions prepared in acetonitrile/water (50/50 v/v) are considered to be stable when stored 208 days between 2 and 8°C.

Table A 129: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 157025 x + 9314.98$, $r = 0.9989$ ($n = 9$)

404 → 344 m/z (Quantification)	$y = 41388.8 x + 1721.84$, $r = 0.9992$ (n = 9)
R230310	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 139470 x + 6576.83$, $r = 0.9989$ (n = 9)
404 → 344 m/z (Quantification)	$y = 35036.6 x + 1647.36$, $r = 0.9992$ (n = 9)

Conclusion

This analytical method for the determination of azoxystrobin and its metabolite R230310 content in honey has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/L for both analytes.

2.1.2.2.3.6.2. Independent laboratory validation

Comments of zRMS:	The analytical method has been independently validated according to the guidance document SANTE/2020/12830 rev.2 for the determination of residues of azoxystrobin and R230310 content in honey. An LOQ of 0.01 mg/kg was confirmed for azoxystrobin and R230310 in honey. The method is acceptable.
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Reference:	KCP 5.2/10
Report	Azoxystrobin – Azoxystrobin - ILV of the Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin and its Metabolite R230310 in Honey Matrices by LC-MS/MS, Homazava N., 2022 Report No. 20210438
Guideline(s):	SANTE/2020/012830 rev1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and method

The objective of the study was to independently validate the analytical residue QuEChERS method described in Report No. 8485926 (KCP 5.2/09) for the determination of azoxystrobin and its metabolite R230310 in honey.

No significant modifications to the primary analytical method were required. 2 g of matrix were transferred into a 50 mL centrifuge tube. 10 mL of purified water and 10 mL of acetonitrile were added and the tube was vigorously shaken by hand for 1 minute. QuEChERS extraction salts were added and the tube was vigorously shaken by hand for 1 minute. The tube was centrifuged at 3500 rpm for 5 minutes. 1 mL of the supernatant was transferred into a centrifuge tube containing 150 mg MgSO₄, 25 mg PSA and 25 mg C18. The tube was manually shaken for 1 minute. The sample was then centrifuged at 3500 rpm for 5 minutes. 400 µL of the purified extract was transferred into a 15 mL centrifuge tube and completed up to 2 mL with purified water. The final extract was analysed for azoxystrobin and its metabolites R230310 using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantification (LOQ) for azoxystrobin and its metabolite R230310 was 0.01 mg/kg.

Analytical conditions

LC conditions

System: HPLC 1290 Infinity II HPLC pump
Autosampler: Agilent 1290 Infinity II multisampler
Column: Acquity BEH C18, 50mm x 2.1mm, 1.7µm
Column temperature: 40°C
Injection volume: 5 µL

Flow: 0.5 mL/min

Mobile phase

Eluent A: 0.2% acetic acid in water

Eluent B: Acetonitrile

Gradient:

Time (min)	% A	% B
0.0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10
5.0	90	10

Retention time:

Azoxystrobin: About 2.55 min

R230310: About 2.33 min

MS conditions

MS system : AB Sciex 6500+ QTrap mass spectrometer

Ionisation type: ESI (ElectroSpray Ionisation)

Polarity: Positive

Scan type : Multiple Reaction Monitoring (MRM)

Azoxystrobin and R230310: 404 → 372 m/z used for quantification and 404 → 344 m/z used for confirmation.

Results and discussions

Table A 130: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (used for Quantification)							
Multi-flower Honey	0.01	114, 111, 115, 111, 112	113	1.6	5	108	4.7
	0.1	107, 100, 104, 104, 104	104	2.2	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (used for Confirmation)							
Multi-flower Honey	0.01	115, 112, 115, 112, 111	113	1.5	5	109	4.6
	0.1	106, 102, 104, 105, 104	104	1.4	5		

Table A 131: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (used for Quantification)							
Multi-flower Honey	0.01	119, 113, 112, 103, 111	112	5.1	5	108	5.2
	0.1	110, 103, 106, 103, 103	105	2.9	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (used for Confirmation)							
Multi-flower Honey	0.01	120, 118, 119, 114, 117	118	2.1	5	112	6.2
	0.1	110, 103, 106, 106, 102	106	2.8	5		

Table A 132: Characteristics for the analytical method used for validation of azoxystrobin and R230310 in honey

	Azoxystrobin	R230310
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). The method is considered highly specific. No residues of azoxystrobin were detected above 30% of the LOQ in any of the control and reagent blank samples, indicating that no interferences were present at the retention time of azoxystrobin in the multi-flower honey. Representative chromatograms representing control and blank samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent are provided. Azoxystrobin mass spectrum is also provided.	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). The method is considered highly specific. No residues of R230310 were detected above 30% of the LOQ in any of the control and reagent blank samples, indicating that no interferences were present at the retention time of R230310 in the multi-flower honey. Representative chromatograms representing control and blank samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent are provided. Azoxystrobin mass spectrum is also 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 performed with 1/x weighing (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A133 below.	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions were calculated by regression analysis performed with 1/x weighing (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A133 below.
Calibration range	Linearity was confirmed over the calibration range 0.1 – 5.0 ng/mL (n = 18), corresponding to a range from 0.0025 – 0.125 mg/kg in matrix which corresponds to 25% of the LOQ to at least 20 % above the highest analyte concentration in the final sample extracts.	Linearity was confirmed over the calibration range 0.1 – 5.0 ng/mL (n = 18), corresponding to a range from 0.0025 – 0.125 mg/kg in matrix which corresponds to 25% of the LOQ to at least 20 % above the highest analyte concentration in the final sample extracts.
Assessment of matrix effects is presented	Yes (insignificant)	Yes (insignificant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for azoxystrobin in multi-flower honey. The LOD is considered as the lowest calibration standard used, 0.1 ng/mL corresponding to 0.0025 mg/kg (25% of the LOQ).	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/kg for R230310 in multi-flower honey. The LOD is considered as the lowest calibration standard used, 0.1 ng/mL corresponding to 0.0025 mg/kg (25% of the LOQ).
Stability of standards and extracts	-	-

Table A 133: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 2303894.2 x + 11386.278$, $r = 0.9994$ (n = 18)
404 → 344 m/z (Quantification)	$y = 1125442.1 x + 1056.9423$, $r = 0.9994$ (n = 18)
R230310	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 1434198.8 x + 11741.859$, $r = 0.9994$ (n = 18)
404 → 344 m/z (Quantification)	$y = 690840.58 x - 439.23296$, $r = 0.9994$ (n = 18)

Conclusion

The primary analytical method presented in KCP 5.2/09 for the determination of azoxystrobin and its metabolite R230310 content in multi-flower honey has been acceptably independently validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/kg for both analytes.

A 2.1.2.2.3.7 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.2.3.8 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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.

Azoxystrobin 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)

A 2.1.2.3.1 Method validation

Comments of zRMS:	<p>The analytical method has been successfully validated according to the guidance documents SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in soil with the LOQ of 0.01 mg/kg.</p> <p>Mean recoveries were in the range of 70 – 120% with relative standard deviations of $\leq 20\%$ for all analytes at each level.</p> <p>The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met.</p> <p>The method is acceptable.</p>
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Reference:	KCP 5.2/11
Report	Validation of an analytical method for Azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in soil, Amic S., 2011, Report N°S11-02190
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Two soil types were used in this validation, sandy loam and loamy silt. Full characterisation is presented in the study report. Soil samples were weighted into a 250 mL polypropylene flask. 100mL of methanol/HCl 1M in ultra pure water (75/25, v/v) were added. Samples were homogenized for 2 hours using a flatbed shaker then were centrifuged for 5 minutes at 3500 rpm. 1 mL of the supernatant was transferred in a polypropylene tube. 9 mL of acetonitrile/ultra pure water (10/90, v/v) were added. Residues were analysed by LC-MS/MS.

The limit of quantification (LOQ) for azoxystrobin, its isomer R230310 and its metabolites R234886, R401553 and R402173 in soil was 0.01 mg/kg.

Analytical conditions

LC conditions

System: LC-MS/MS API 4000 (Sciex)

Automatic sampler SIL20AC (Shimadzu)

Pump LC20AD (Shimadzu)

Column oven: CTO-20AC (Shimadzu)

Column: AIT France Kromasil KR100 5C18, 50 mm x 3.0 mm, 5µm

Column temperature: 40°C

Automatic sampler temperature: 4°C

Flow: 1 mL/min

Mobile phase

Eluent A: Acetonitrile

Eluent B: Water with 0.2% v/v acetic acid

Gradient:

Time (min)	% A	% B
0	10	90
1.0	50	50
2.5	50	50
3.0	90	10
3.5	90	10
4.0	10	90
5.0	10	90

Divert valve:

From 0.0 min to 0.8 min to waste

From 0.8 min to MS

Injection volume: 50 and 75 µL

Retention time:

Azoxystrobin: About 2.6 min

R230310: About 2.3 min

R234886: About 1.94 min

R401553: About 1.29 min

R402173: About 1.9 min

MS conditions

Ionisation type: Electrospray ionisation (ESI⁺)

Polarity: Positive ion mode

Acquisition mode: MRM

Azoxystrobin and R230310: 404 → 372 proposed for quantification and 404 → 344 proposed for confirmation.

R234886: 390 → 372 m/z proposed for quantification and 390 → 344 m/z proposed for confirmation.

R401553: 214 → 187 m/z proposed for quantification and 214 → 102 m/z proposed for confirmation.

R402173: 334 → 316 m/z proposed for quantification and 334 → 188 m/z proposed for confirmation.

Results and discussions

Table A 134: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (µg/L mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Soil 1 (Sandy loam)	0.01	86, 86, 86, 88, 88	87	1	5	88	4
	0.1	90, 97, 88, 87, 88	90	5	5		
Soil 2 (Loamy silt)	0.01	126, 116, 116, 125, 116	120	4	5	116	7
	0.1	103, 126, 116, 107, 109	112	8	5		

Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Confirmation)							
Soil 1 (Sandy loam)	0.01	78, 81, 84, 83, 85	82	3	5	86	6
	0.1	91, 96, 86, 89, 88	90	4	5		
Soil 2 (Loamy silt)	0.01	103, 96, 88, 98, 89	95	7	5	104	12
	0.1	105, 127, 118, 111, 112	114	7	5		

Table A 135: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (μg/L mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)							
Soil 1 (Sandy loam)	0.01	90, 92, 87, 86, 89	89	3	5	89	3
	0.1	91, 96, 87, 89, 88	90	4	5		
Soil 2 (Loamy silt)	0.01	86, 98, 94, 94, 90	92	5	5	101	11
	0.1	100, 125, 112, 108, 106	110	8	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Confirmation)							
Soil 1 (Sandy loam)	0.01	86, 88, 89, 91, 83	87	4	5	88	4
	0.1	86, 94, 85, 87, 88	88	4	5		
Soil 2 (Loamy silt)	0.01	124, 102, 115, 124, 118	117	8	5	116	7
	0.1	108, 129, 117, 107, 114	115	8	5		

Table A 136: Recovery results from method validation of R234886 using the analytical method

R234886							
Matrix	Fortification Level ($\mu\text{g/L}$ mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 390 \rightarrow 372 <i>m/z</i> (Proposed for Quantification)							
Soil 1 (Sandy loam)	0.01	86, 88, 84, 89, 86	86	2	5	85	3
	0.1	84, 84, 81, 84, 81	83	2	5		
Soil 2 (Loamy silt)	0.01	122, 110, 108, 120, 112	115	5	5	112	7
	0.1	100, 124, 112, 102, 105	109	9	5		
Transition <i>m/z</i> 390 \rightarrow 344 <i>m/z</i> (Proposed for Confirmation)							
Soil 1 (Sandy loam)	0.01	90, 90, 92, 85, 92	90	3	5	86	6
	0.1	85, 83, 83, 80, 83	83	2	5		
Soil 2 (Loamy silt)	0.01	121, 106, 93, 126, 123	114	12	5	112	10
	0.1	103, 125, 114, 103, 111	111	8	5		

Table A 137: Recovery results from method validation of R401553 using the analytical method

R401553							
Matrix	Fortification Level ($\mu\text{g/L}$ mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 214 → 187 <i>m/z</i> (Proposed for Quantification)							
Soil 1 (Sandy loam)	0.01	95, 95, 88, 85, 88	90	5	5	89	4
	0.1	91, 93, 87, 87, 86	89	4	5		
Soil 2 (Loamy silt)	0.01	115, 114, 100, 124, 112	113	8	5	110	8
	0.1	99, 121, 111, 99, 104	107	9	5		
Transition <i>m/z</i> 214 → 120 <i>m/z</i> (Proposed for Confirmation)							
Soil 1 (Sandy loam)	0.01	79, 79, 79, 80, 84	80	3	5	82	4
	0.1	87, 88, 80, 85, 84	85	4	5		
Soil 2 (Loamy silt)	0.01	139, 110, 111, 112, 109	116	11	5	112	11
	0.1	99, 126, 107, 104, 100	107	10	5		

Table A 138: Recovery results from method validation of R402173 using the analytical method

R402173							
Matrix	Fortification Level (µg/L mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 334 → 316 <i>m/z</i> (Proposed for Quantification)							
Soil 1 (Sandy loam)	0.01	89, 87, 86, 86, 89	88	2	5	86	3
	0.1	87, 85, 83, 85, 82	84	2	5		
Soil 2 (Loamy silt)	0.01	128, 113, 114, 119, 121	119	5	5	114	8
	0.1	101, 121, 112, 103, 106	109	7	5		
Transition <i>m/z</i> 334 → 188 <i>m/z</i> (Proposed for Confirmation)							
Soil 1 (Sandy loam)	0.01	79, 81, 90, 99, 91	88	9	5	87	6
	0.1	88, 88, 82, 87, 85	86	3	5		
Soil 2 (Loamy silt)	0.01	127, 125, 96, 116, 113	115	11	5	113	9
	0.1	102, 123, 112, 105, 108	110	7	5		

Table A 139: Characteristics for the analytical method used for validation of azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in soil

	Azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173
Specificity	<p>LC-MS/MS determination was conducted by monitoring two mass transitions (Azoxystrobin and R230310: 404 \rightarrow 372 m/z for quantification and 404 \rightarrow 344 m/z for confirmation ; R234886: 390 \rightarrow 372 m/z for quantification and 390 \rightarrow 344 m/z for confirmation ; R401553: 214 \rightarrow 187 m/z for quantification and 214 \rightarrow 120 m/z for confirmation ; R402173: 334 \rightarrow 316 m/z for quantification and 334 \rightarrow 188 m/z for confirmation).</p> <p>A reagent blank prepared according to the analytical method and the unfortified soil specimens showed no significant interference (< 30% of LOQ) at the retention times of the analytes. The method is specific.</p> <p>Representative chromatograms for each soil matrix and analyte representing control samples, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank extracts are provided.</p>
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 performed with 1/x weighting (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A140 below.
Calibration range	Linearity was confirmed over the calibration range 0.025 – 5.0 ng/mL ($n = 7$ or 8) corresponding to a range from 0.0025 – 0.5 mg/kg in sample which corresponds to more than 30% of the LOQ and at least + 20% above the highest level of recovery validated.
Assessment of matrix effects is presented	Yes (Unsignificant)
Limit of determination/quantification	The LOQ is defined as the lowest analyte concentration at which the methodology had been successfully validated. The LOQ is 0.01 mg/kg for all analytes in soil.
Stability of standards and extracts	The final extracts fortified at the LOQ level (0.01 mg/kg) were considered to be stable when stored between 0 °C to 9 °C for 9 days.

Table A 140 Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 \rightarrow 372 m/z (Quantification)	$y = 1261128 x + 10901$, $r = 0.9991$ ($n = 8$)
404 \rightarrow 344 m/z (Confirmation)	$y = 191419 x + 2492$, $r = 0.9990$ ($n = 8$)

R230310	
Transition	Linearity data
404 \rightarrow 372 m/z (Quantification)	$y = 798847 x + 5072$, $r = 0.9987$ ($n = 7$)
404 \rightarrow 344 m/z (Confirmation)	$y = 122534 x + 623$, $r = 0.9986$ ($n = 7$)

R234886	
Transition	Linearity data
390 → 372 m/z (Quantification)	$y = 305345 x + 1796$, $r = 0.9997$ (n = 8)
390 → 344 m/z (Confirmation)	$y = 46522 x + 100$, $r = 0.9998$ (n = 8)

R401553	
Transition	Linearity data
214 → 187 m/z (Quantification)	$y = 30682 x + 282$, $r = 0.9994$ (n = 8)
214 → 120 m/z (Confirmation)	$y = 25101 x + 341$, $r = 0.9998$ (n = 8)

R402173	
Transition	Linearity data
334 → 316 m/z (Quantification)	$y = 187603 x + 392$, $r = 0.9997$ (n = 8)
334 → 188 m/z (Confirmation)	$y = 39220 x + 175$, $r = 0.9998$ (n = 8)

Conclusion

This analytical method for the content determination of azoxystrobin, its isomer R230310 and its metabolites R234886, R401553 and R402173 in soil (Sandy loam and Loamy silt) has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.01 mg/kg for all analytes.

A 2.1.2.3.1.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.3.1.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

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

A 2.1.2.4.1 Method validation

Comments of zRMS:	<p>The study of Krebber, R., Sandau, C., 2015 on modification M002 of analytical method 01387 for the determination of various pesticides in drinking and surface water by HPLC-MS/MS has been evaluated in Registration Report for ADM.03500.F.2.B (Soratel) on November 2022 by zRMS-PL and the summary is presented below.</p> <p>The analytical method 01387/M002 for the determination of concentrations of prothioconazole and prothioconazole-desthio in surface water by HPLC-MS/MS using two MRM transitions has been validated.</p> <p>The limit of quantitation (LOQ) is 0.05 µg/L for all analytes in surface water.</p> <p>Because of the direct measurement of the samples recovery rates cannot be calculated. The relative standard deviations for the peak areas were ≤ 20% for all analytes and MRM transitions.</p> <p>The method meets all guideline criteria to determine concentrations in drinking and surface water of prothioconazole and prothioconazole-desthio at 0.05 µg/L.</p> <p><u>Remark:</u></p> <p>A validated method for drinking water is not necessary since the limit of quantitation for surface water is equal or below the drinking water limit of 0.1 µg/L.</p>
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Reference: KCP 5.2/12

Report	Modification M002 of analytical method 01387 for the determination of various pesticides in drinking and surface water by HPLC-MS/MS, Krebber, R.; Sandau, C., 2015, Report N°M-526061-01-1
Guideline(s):	SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Analytes were determined in water (drinking water and surface water) using a liquid chromatography technique (LC-MS/MS, 2 MRM transitions). The limit of quantification (LOQ) for prothioconazole and its metabolite prothioconazole-desthio was 0.05 µg/L. A validation for drinking water was not necessary because the limit of quantitation for surface water is equal or below the drinking water limit of 0.1 µg/L. Water samples were determined by direct injection into the HPLC-MS/MS instrument using the positive ion mode for all analytes without further clean-up. Concentrations were quantified using external matrix-matched standard solutions.

Analytical conditions

System: LC-MS/MS

Retention time:

Prothioconazole: 2.91 min

Prothioconazole-desthio: 2.84 min

MS conditions

Compound		Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell time [msec]	Collision Energy (eV)	Polarity
Prothioconazole	Quantitation	344	189	10	29	positive
	Confirmation	344	154	10	39	positive
Prothioconazole -desthio	Quantitation	312	70	10	25	positive
	Confirmation	312	125	10	35	positive

Results and discussions

For method validation surface water (+ 50 mg cysteine hydrochloride / L) / formic acid (1000/0.1 v/v) was fortified with prothioconazole and its metabolite JAU 6476-desthio. Fortification levels were 0.05 µg/L (LOQ) and 0.5 µg/L (10*LOQ).

From each test solution five aliquots were taken and injected twice into the HPLC-MS/MS instrument. The peak areas and retention times for prothioconazole and JAU 6476-desthio in surface water were determined and are listed in the tables below. Because of the direct measurement of the samples recovery rates cannot be calculated. Thus, precision data are presented.

Table A 141: Recovery results from method validation of prothioconazole using the analytical method

Matrix	Fortification Level (µg/L)	Peak area (single values) (area counts)	Mean (area count)	Rel. Std. Dev. (%)	Replicates
Transition m/z 344 → 189 m/z (Proposed for Quantification)					
Surface water	0.05	8645, 8204, 8566, 8859, 8738 8741, 8859, 8691, 8636, 8859	8680	2.3	10
	0.5	89774, 85561, 85395, 85405, 89321 85820, 89712, 88393, 89082, 89505	87797	2.3	10
Transition m/z 344 → 154 m/z (Proposed for Confirmation)					
Surface water	0.05	6790, 6771, 6958, 6364, 6920 6207, 6413, 5472, 5755, 5336	6299	9.5	10
	0.5	68113, 67347, 70861, 76320, 68686 67232, 69030, 69063, 70477, 70946	69808	3.8	10

Table A 142: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification Level (µg/L)	Peak area (single values) (area counts)	Mean (area count)	Rel. Std. Dev. (%)	Replicates
Transition m/z 312 → 70 m/z (Proposed for Quantification)					
Surface water	0.05	155867, 151051, 152289, 148150, 145810 153369, 151896, 148989, 151847, 151105	151037	1.9	10
	0.5	1511351, 1514428, 1556334, 1524425, 1533506 1500634, 1523083, 1542504, 1506524, 1509210	1522200	1.2	10
Transition m/z 312 → 125 m/z (Proposed for Confirmation)					
Surface water	0.05	94174, 93527, 92626, 92165, 91693 92026, 95671, 93143, 93830, 91886	93164	1.6	10
	0.5	950877, 938876, 949687, 943186, 921905 916213, 935352, 938690, 912477, 915328	932259	1.6	10

Table A 143: Characteristics for the analytical method used for validation of prothioconazole and its metabolite prothioconazole-desthio in water

	Prothioconazole and its metabolite Prothioconazole-desthio
Specificity	<p>LC-MS/MS determination was conducted by monitoring two mass transitions (Prothioconazole: 344 → 189 m/z for quantification and 344 → 154 m/z for confirmation ; Prothioconazole-desthio: 312 → 70 m/z for quantification and 312 → 125 m/z for confirmation).</p> <p>No signals/peaks interfering with the detection of the analytes were observed in solutions of untreated control specimens. Apparent concentrations in control samples were below $0.3 \times \text{LOQ}$.</p> <p>The method is highly specific.</p> <p>Representative chromatograms for each matrix and analyte representing control samples, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank extracts are provided.</p>
Calibration (type, number of data points)	<p>The linearity of the method was demonstrated using non-matrix matched calibration standards. Linear calibration functions performing with 1/x weighting were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99.</p> <p>Please see table A144 below.</p>
Calibration range	<p>Prothioconazole: Linearity was confirmed over the calibration range 0.015 – 10.0 µg/L corresponding to a range from 0.015 – 10.0 µg/L in sample extract which corresponds to more than 30% of the LOQ and at least + 20% above the highest level of recovery validated.</p> <p>Prothioconazole-desthio: Linearity was confirmed over the calibration range 0.015 – 1.0 µg/L corresponding to a range from 0.015 – 1.0 µg/L in sample extract which corresponds to more than 30% of the LOQ and at least + 20% above the highest level of recovery validated.</p>
Assessment of matrix effects is presented	Yes (Unsignificant)
Limit of determination/quantification	<p>The LOQ is defined as the lowest analyte concentration at which the methodology had been successfully validated. The LOQ is 0.05 µg/L for all analytes in surface water</p> <p>The LOD is considered as the lowest calibration standard used, 0.015 µg/L for all analytes.</p>
Stability of standards and extracts	Results of the test on storage stability show that prothioconazole-desthio is stable for a storage period of at least 7 days under freezer conditions at $\leq -18^\circ\text{C}$. Prothioconazole showed a significant depletion. Prothioconazole can be stabilized by addition of cysteine hydrochloride to the water sample.

Table A 144 Linearity of detector response

Prothioconazole	
Transition	Linearity data
344 → 189 m/z (Quantification)	$y = 1.7994\text{E}+05 \cdot x - 225.59$
344 → 154 m/z (Confirmation)	$y = 1.428\text{E}+05 \cdot x - 659.32$

Prothioconazole-desthio	
Transition	Linearity data
312 → 125 m/z (Quantification)	$y=2.9741E+06*x + 5603$
312 → 70 m/z (Confirmation)	$y=1.7841E+05*x + 5882.5$

Conclusion

The analytical method successfully validated for the determination of prothioconazole and its metabolite prothioconazole-desthio. The method complies with all criteria according to SANTE/2020/12830, Rev 2 and is therefore suitable as an enforcement method for the determination of prothioconazole and its metabolite prothioconazole-desthio in drinking and surface water by HPLC-MS/MS.

A 2.1.2.4.2 Independent laboratory validation

Comments of zRMS:	<p>The study of Thies, S., 2015 on independent laboratory validation of the BCS analytical method 01387/M002 for the determination of various pesticides in surface water by HPLC-MS/MS has been evaluated in Registration Report for ADM.03500.F.2.B (Soratel) on November 2022 by zRMS-PL and the summary is presented below.</p> <p>The analytical BCS method 01387/M002 for the determination of concentrations of prothioconazole and prothioconazole-desthio in surface water by HPLC-MS/MS using two MRM transitions has been independently validated.</p> <p>The limit of quantitation (LOQ) for all analytes is 0.05 µg/L in surface water.</p> <p>The relative standard deviations for the peak areas were ≤ 20% for all MRM transitions of all analytes.</p> <p>The method meets all guideline criteria to determine concentrations in surface water of the described analytes at 0.05 µg/L.</p>
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Reference:	KCP 5.2/13
Report	Independent laboratory validation of the BCS analytical method 01387/M002 for the determination of various pesticides in surface water by HPLC-MS/MS, Thies S., 2015, Report N°M-536990-01-1
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to independently validate a method as described in study number M-526061-01-1 for the determination of prothioconazole and prothioconazole-desthio in surface water.

The samples are added with of 0.1 mL formic acid per litre sample volume and directly injected into the HPLC or after appropriate dilution with a mixture of river Rhine water / formic acid (1000 / 0.1, v/v). For stabilisation of prothioconazole, cysteine hydrochloride (50 mg/L) has to be added. Water samples were determined by direct injection into the HPLC-MS/MS instrument using the positive ion mode for two transitions for all analytes without further clean-up. Concentrations were quantified using external matrix-matched standard solutions. The limit of quantification is 0.05 µg/L for both analytes.

Analytical conditions

LC conditions

System: LC-MS/MS

Retention time:

Prothioconazole: 2.02 min

Prothioconazole-desthio: 1.99 min

MS conditions

MRM transitions

Prothioconazole: m/z 344 Da → m/z 189 Da for quantitation and m/z 344 Da → m/z 154 Da for confirmation.

Prothioconazole-desthio: m/z 312 Da → m/z 70 Da for quantitation and m/z 312 Da → m/z 125 Da for confirmation

Results and discussions

For method validation surface water was fortified with a mixture of the described analytes (fortification levels 0.05 µg/L (LOQ) and 0.5 µg/L (10*LOQ)).

From each test solution five aliquots were taken and injected into the HPLC-MS/MS instrument. The peak areas and retention times of the MRM transitions for the analytes in surface water were determined. As a measure for the precision of the method, the intra-laboratory repeatability is given as relative standard deviation (% RSD) for surface water samples at fortification levels of 0.05 µg/L and 0.5 µg/L for both MRM transitions.

Table A 145: Recovery results from method validation of prothioconazole using the analytical method

Matrix	Fortification Level (µg/L)	Peak area (single values) (area counts)	Mean (area count)	Rel. Std. Dev. (%)	Replicates
Transition m/z 344 → 189 m/z (Proposed for Quantification)					
Surface water	0.05	7510, 6130, 7360, 7310, 7340	7130	7.9	5
	0.5	74700, 62000, 77300, 75600, 71800	72280	8.4	5
Transition m/z 344 → 154 m/z (Proposed for Confirmation)					
Surface water	0.05	4010, 5080, 4750, 5020, 4430	4658	9.5	5
	0.5	56600, 53400, 56200, 53800, 53800	54760	2.8	5

Table A 146: Recovery results from method validation of prothioconazole-desthio using the analytical method

Matrix	Fortification Level (µg/L)	Peak area (single values) (area counts)	Mean (area count)	Rel. Std. Dev. (%)	Replicates
Transition m/z 312 → 70 m/z (Proposed for Quantification)					
Surface water	0.05	71900, 70300, 59600, 71700, 73100	69320	8.0	5
	0.5	682000, 69100, 694000, 690000, 694000	690200	0.7	5
Transition m/z 312 → 125 m/z (Proposed for Confirmation)					
Surface water	0.05	49600, 53400, 53100, 52300	51380	4.3	5
	0.5	606000, 462000, 523000, 514000, 481000	517200	11	5

Table A 147: Characteristics for the analytical method used for validation of prothioconazole and prothioconazole-desthio in water

	Prothioconazole and prothioconazole-desthio
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (Prothioconazole: 344 → 189 m/z for quantification and 344 → 154 m/z for confirmation ; Prothioconazole-desthio: 312 → 70 m/z for quantification and 312 → 125 m/z for confirmation). Apparent concentrations in control samples were below 0.3 × LOQ. No signals/peaks interfering with the detection of the analytes were observed in solutions of untreated control specimens. Representative chromatograms for each analyte representing control samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ are provided, together with a product ion spectrum.
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched calibration standards. Linear calibration functions performing with 1/x weighted were calculated by regression analysis (regression model: y = a*x + b). The correlation coefficients, r obtained were > 0.99. Please see table A148 below.

	Prothioconazole and prothioconazole-desthio
Calibration range	Linearity was confirmed over the calibration range 0.015 – 1.0 µg/mL, which corresponds to 30% of the LOQ at least + 20 % of the highest analyte concentration level.
Assessment of matrix effects is presented	Yes (not significant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.05 µg/L for both analytes in surface water. The limit of detection (LOD) is 0.015 µg/L for prothioconazole and its metabolite prothioconazole-desthio in surface water (corresponding to the lowest calibration standard).
Stability of standards and extracts	NA

Table A 148: Linearity of detector response

Prothioconazole	
Transition	Linearity data
344 → 189 m/z (Quantification)	y= 1.66e+005 x – 994, r = 0.9961
344 → 154 m/z (Confirmation)	y= 1.39e+005 x + -1.56e+003, r = 0.9971

Prothioconazole-desthio	
Transition	Linearity data
312 → 125 m/z (Quantification)	y=1.17e+006 x + 2.54e+004, r = 0.9987
312 → 70 m/z (Confirmation)	y= 6.9e+005 x + 1.17e+004, r = 0.9992

Conclusion

This analytical method was independently validated for the determination of prothioconazole and its metabolite prothioconazole-desthio. The method complies with all criteria for independent laboratory validation according to SANTE/2020/12830, Rev 2 and is therefore suitable as an enforcement method for the determination of prothioconazole and its metabolite prothioconazole-desthio in drinking and surface water by HPLC-MS/MS.

A 2.1.2.4.3 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.4.4 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 2, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.2.4.5 Method validation

Comments of zRMS:	The analytical method has been successfully validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in water with the LOQ of 0.1 µg/L. Mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met. The method is acceptable.
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Reference: KCP 5.2/14

Report Validation of an analytical method for Azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in water, Amic S., 2011,

Report N°S11-02191

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Analytes were determined in water (drinking water, surface water and groundwater) using a liquid chromatography technique (LC-MS/MS, 2 mass transitions monitored). The limit of quantification (LOQ) for azoxystrobin, its isomer R230310 and its metabolites R234886, R401553 and R402173 in water was 0.1 µg/L.

Residues were directly analysed by LC-MS/MS.

Analytical conditions

LC conditions

System: LC-MS/MS API 4000 (Sciex)
Automatic sampler SIL20AC (Shimadzu)
Pump LC20AD (Shimadzu)
Column oven: CTO-20AC (Shimadzu)

Column: AIT France Kromasil KR100 5C18, 50 mm x 3.0 mm, 5µm

Column temperature: 40°C

Automatic sampler temperature: 4°C

Flow: 1mL/min

Mobile phase

Eluent A: Acetonitrile

Eluent B: Water with 0.2% v/v acetic acid

Gradient:

Time (min)	% A	% B
0	10	90
1.0	50	50
2.5	50	50
3.0	90	10
3.5	90	10
4.0	10	90
5.0	10	90

Divert valve:

From 0.0 min to 0.8 min to waste

From 0.8 min to MS

Injection volume: 10, 50 and 100 µL

Retention time:

Azoxystrobin: About 2.6 min

R230310: About 2.3 min

R234886: About 1.95 min

R401553: About 1.3 min

R402173: About 1.9 min

MS conditions

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Acquisition mode: MRM

Azoxystrobin and R230310: 404 → 372 proposed for quantification and 404 → 344 proposed for confirmation.

R234886: 390 → 372 m/z proposed for quantification and 390 → 344 m/z proposed for confirmation.
R401553: 214 → 187 m/z proposed for quantification and 214 → 102 m/z proposed for confirmation.
R402173: 334 → 316 m/z proposed for quantification and 334 → 188 m/z proposed for confirmation.

Results and discussions

Table A 142 9: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Drinking water	0.1	71, 78, 76, 76, 79	76	4	5	77	4
	1.0	79, 74, 79, 79, 79	78	3	5		
Surface water	0.1	81, 79, 82, 77, 83	81	3	5	80	3
	1.0	78, 77, 81, 79, 78	78	2	5		
Groundwater	0.1	83, 81, 80, 83, 80	82	2	5	79	5
	1.0	80, 78, 81, 69, 78	77	6	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Drinking water	0.1	81, 65, 80, 83, 79	78	9	5	78	7
	1.0	78, 73, 77, 78, 80	77	3	5		
Surface water	0.1	87, 88, 84, 79, 76	83	6	5	80	6
	1.0	76, 77, 80, 79, 76	77	3	5		
Groundwater	0.1	81, 84, 79, 78, 73	79	5	5	77	5
	1.0	76, 79, 76, 70, 79	76	5	5		

Table A 142 150: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Drinking water	0.1	78, 85, 83, 88, 79	82	7	5	81	5
	1.0	78, 78, 81, 82, 83	80	3	5		
Surface water	0.1	82, 79, 84, 86, 87	83	4	5	82	4
	1.0	80, 79, 82, 79, 82	80	2	5		
Groundwater	0.1	84, 90, 84, 84, 87	86	3	5	82	8
	1.0	79, 81, 81, 66, 80	77	8	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Drinking water	0.1	88, 95, 81, 70, 94	86	12	5	84	9
	1.0	83, 81, 86, 79, 81	82	3	5		
Surface water	0.1	90, 83, 97, 79, 86	87	8	5	83	7
	1.0	79, 80, 81, 79, 80	80	1	5		
Groundwater	0.1	80, 81, 75, 82, 86	81	5	5	80	7
	1.0	81, 80, 85, 68, 79	79	8	5		

Table A 143 151: Recovery results from method validation of R234886 using the analytical method

R234886							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 390 → 372 m/z (Proposed for Quantification)							
Drinking water	0.1	92, 90, 96, 92, 90	92	3	5	92	2
	1.0	93, 92, 93, 91, 91	92	1	5		
Surface water	0.1	94, 93, 94, 98, 91	94	3	5	93	3
	1.0	92, 92, 91, 93, 89	91	2	5		
Groundwater	0.1	92, 86, 88, 85, 83	87	4	5	85	3

	1.0	87, 84, 83, 83, 84	84	2	5		
Transition m/z 390 → 344 m/z (Proposed for Confirmation)							
Drinking water	0.1	91, 92, 99, 97, 90	94	4	5	94	4
	1.0	95, 96, 96, 90, 98	95	3	5		
Surface water	0.1	90, 95, 89, 89, 91	91	3	5	91	3
	1.0	96, 91, 90, 92, 87	91	4	5		
Groundwater	0.1	86, 85, 84, 93, 87	87	4	5	86	4
	1.0	88, 86, 84, 82, 81	84	3	5		

Table A 144-152: Recovery results from method validation of R401553 using the analytical method

R401553							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 214 → 187 m/z (Proposed for Quantification)							
Drinking water	0.1	86, 90, 95, 92, 96	92	5	5	92	4
	1.0	90, 97, 91, 90, 96	93	4	5		
Surface water	0.1	106, 98, 92, 94, 99	98	5	5	95	6
	1.0	93, 92, 97, 96, 84	95	6	5		
Groundwater	0.1	93, 95, 89, 92, 98	93	4	5	94	3
	1.0	94, 90, 98, 95, 97	95	3	5		
Transition m/z 214 → 120 m/z (Proposed for Confirmation)							
Drinking water	0.1	96, 87, 109, 103, 95	98	9	5	97	6
	1.0	94, 98, 97, 99, 94	96	2	5		
Surface water	0.1	89, 106, 90, 109, 109	100	10	5	97	9
	1.0	97, 93, 93, 97, 84	93	6	5		
Groundwater	0.1	96, 78, 88, 87, 98	90	9	5	93	7
	1.0	98, 98, 97, 95, 98	97	1	5		

Table A 144-153: Recovery results from method validation of R402173 using the analytical method

R402173							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 334 → 316 <i>m/z</i> (Proposed for Quantification)							
Drinking water	0.1	97, 96, 97, 94, 96	96	1	5	96	1
	1.0	96, 94, 96, 96, 95	96	1	5		
Surface water	0.1	93, 97, 96, 98, 90	94	3	5	94	3
	1.0	96, 96, 92, 95, 90	94	3	5		
Groundwater	0.1	89, 92, 97, 90, 91	91	3	5	90	3
	1.0	88, 86, 88, 88, 87	88	1	5		
Transition <i>m/z</i> 334 → 188 <i>m/z</i> (Proposed for Confirmation)							
Drinking water	0.1	92, 88, 90, 96, 99	93	5	5	94	3
	1.0	95, 94, 95, 94, 94	95	1	5		
Surface water	0.1	89, 91, 99, 93, 90	92	4	5	94	4
	1.0	98, 97, 96, 96, 91	96	3	5		
Groundwater	0.1	94, 93, 88, 85, 85	89	5	5	88	4
	1.0	90, 87, 88, 88, 84	88	2	5		

Table A 144-154: Characteristics for the analytical method used for validation of azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173 in water

Azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173	
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (Azoxystrobin and R230310: 404 → 372 m/z for quantification and 404 → 344 m/z for confirmation ; R234886: 390 → 372 m/z for quantification and 390 → 344 m/z for confirmation ; R401553:

	Azoxystrobin, its isomer R230310 and metabolites R234886, R401553 and R402173
	<p>214 → 187 m/z for quantification and 214 → 120 m/z for confirmation ; R402173: 334 → 316 m/z for quantification and 334 → 188 m/z for confirmation).</p> <p>A reagent blank prepared according to the analytical method and the unfortified water (drinking water, surface water and groundwater) specimens showed no significant interference (< 30% of LOQ) at the retention times of the analytes. The method is highly specific.</p> <p>Representative chromatograms for each matrix and analyte representing control samples, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank extracts are provided.</p>
Calibration (type, number of data points)	The linearity of the method was demonstrated using non-matrix matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A147 A155 below.
Calibration range	Linearity was confirmed over the calibration range 0.025 – 5.0 µg/L (n = 8), corresponding to a range from 0.025 – 5.0 µg/L in sample extract which corresponds to more than 30% of the LOQ and at least + 20% above the highest level of recovery validated.
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	<p>The LOQ is defined as the lowest analyte concentration at which the methodology had been successfully validated. The LOQ is 0.1 µg/L for all analytes in water (drinking water, surface water and groundwater).</p> <p>The LOD is considered as the lowest calibration standard used, 0.025 µg/L for all analytes in water (drinking water, surface water and groundwater).</p>
Stability of standards and extracts	The final extracts fortified at the LOQ level (0.1 µg/L) were considered to be stable when stored between 0 °C to 9 °C for 9 days in the dark.

Table A 147 A155: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 198412 x + 1652, r = 0.9998 (n = 8)$
404 → 344 m/z (Confirmation)	$y = 30380 x + 256, r = 0.9999 (n = 8)$
R230310	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 129710 x + 304, r = 0.9997 (n = 8)$
404 → 344 m/z (Confirmation)	$y = 19189 x + 111, r = 0.9999 (n = 8)$
R234886	
Transition	Linearity data
390 → 372 m/z (Quantification)	$y = 399760 x + 2201, r = 0.9998 (n = 8)$
390 → 344 m/z (Confirmation)	$y = 59668 x + 296, r = 0.9996 (n = 8)$
R401553	
Transition	Linearity data
214 → 187 m/z (Quantification)	$y = 39700 x + 226, r = 0.9994 (n = 8)$
214 → 120 m/z (Confirmation)	$y = 31397 x + 208, r = 0.9995 (n = 8)$
R402173	
Transition	Linearity data
334 → 316 m/z (Quantification)	$y = 249268 x + 1575, r = 0.9997 (n = 8)$
334 → 188 m/z (Confirmation)	$y = 53578 x + 521, r = 0.9994 (n = 8)$

Conclusion

This analytical method for the content determination of azoxystrobin, its isomer R230310 and its metabolites R234886, R401553 and R402173 in water (drinking water, surface water and groundwater) has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.1 µg/L for all analytes.

A 2.1.2.4.6 Independent laboratory validation

Comments of zRMS:	The analytical method (study number S11-02191) has been independently validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin and R230310 content in water. An LOQ of 0.1 µg/L was confirmed for azoxystrobin and R230310 in drinking water. Mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met. The method is acceptable.
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Reference:	KCP 5.2/4315
Report	Independent Laboratory validation of a method for the determination of Azoxystrobin and R230310 in water, Siekmann D., 2017, Report N°S17-01575
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to independently validate a method as described in study number S11-02191 for the determination of azoxystrobin and its Z-isomer R230310 in drinking water.

Samples of drinking water were fortified with the analytical standards of azoxystrobin and R230310 and injected directly into the HPLC-MS/MS system. The limit of quantification is 0.1 µg/L for both analytes.

Analytical conditions

LC conditions

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

Pre-column: HPLC guard column with 4mm C18 cartridge

Column: MZ-Analysentechnik Kromasil 100-5 C18, 50mm x 3.0mm, 5µm

Column temperature: 40°C

Flow: 1 mL/min

Mobile phase

Eluent A: Water containing 0.2% (v/v) acetic acid

Eluent B: Acetonitrile

Gradient:

Time (min)	% A	% B
0.0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10
5.0	90	10
5.1	90	10

Divert valve:

0.0 min to 1.0 min to waste
1.0 min to 3.5 min to MS
3.5 min to 5.1 min to waste
Injection volume: 50 µL
Retention time:
Azoxystrobin: About 2.3 min
R230310: About 2.0 min

MS conditions

MS system: SCIEX API 5500

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Capillary voltage: 5500V

Ionspray turbo heater: 400°C

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

Mass transitions (azoxystrobin and R230310): 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 149 156: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Drinking water	0.1	101, 101, 102, 102, 103	102	1	5	100	3
	1	101, 98, 99, 94, 95	97	3	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Drinking water	0.1	101, 100, 102, 102, 104	102	1	5	100	3
	1	101, 98, 100, 96, 95	98	3	5		

Recoveries are without any blank correction

Table A 149 157: Recovery results from method validation of R230310 using the analytical method

R230310							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Drinking water	0.1	95, 96, 98, 97, 100	97	2	5	98	2
	1	100, 99, 100, 95, 95	98	3	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Drinking water	0.1	97, 97, 96, 98, 100	98	2	5	98	2
	1	100, 100, 102, 96, 95	98	3	5		

Recoveries are without any blank correction

Table A 150 158: Characteristics for the analytical method used for validation of azoxystrobin and R230310 in water

Azoxystrobin and R230310	
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). A reagent blank and two control samples of drinking water were extracted and analysed according to the method. For both mass transition, the samples showed no significant interference (above 30% of LOQ) at the retention time of the analytes in drinking water. The method is highly specific.

	Azoxystrobin and R230310
	Representative chromatograms for each analyte representing control samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ are provided, together with a product ion spectrum.
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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A15-9 below.
Calibration range	Linearity was confirmed over the calibration range 0.025 – 5.0 ng/mL ($n = 8$), which corresponds to no more than 30% of the LOQ at least + 20 % of the highest analyte concentration level.
Assessment of matrix effects is presented	Yes (not significant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.1 µg/L for both analytes in drinking water. The LOD is considered as the lowest calibration standard used (0.025 ng/mL), equivalent to 0.25 µg/L for all analytes in drinking water.
Stability of standards and extracts	NA

Table A 15-9: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 1.12 \cdot 10^6 x - 258$, $r = 0.9994$ ($n = 8$)
404 → 344 m/z (Quantification)	$y = 3.33 \cdot 10^6 x + 2.39 \cdot 10^3$, $r = 0.9991$ ($n = 8$)

R230310	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 9.0 \cdot 10^5 x + 1.84 \cdot 10^3$, $r = 0.9992$ ($n = 8$)
404 → 344 m/z (Quantification)	$y = 2.64 \cdot 10^6 x + 6.19 \cdot 10^3$, $r = 0.9989$ ($n = 8$)

Conclusion

This analytical method for the determination of azoxystrobin and R230310 content in drinking water has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.1 µg/L for azoxystrobin and R230310.

A 2.1.2.4.7 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.4.8 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

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

Comments of zRMS:	The analytical method has been successfully validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin in air with the LOQ of 2.2 µg/filter for azoxystrobin (equivalent to 0.003 µg/m ³ air aspired).. Mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20%. The acceptance criteria of the SANTE/2020/12830 rev.2 for the analytical method were met.
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The method is acceptable.

A 2.1.2.5.1

Method validation

Reference: KCP 5.2/1416

Report Azoxystrobin: Validation of an analytical method for azoxystrobin in air, Amic S., 2011, Report N°S11-02192

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

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to validate an analytical method using two mass transitions for the determination of azoxystrobin in air by LC-MS/MS technique.

Residues were extracted with acetonitrile. An aliquote was diluted in acetonitrile/water (10/90 v/v). Final extrac were injected directly into the HPLC-MS/MS system. The limit of quantification is 2.2 µg/filter (equivalent to 0.003 µg/m³ air aspired).

Analytical conditions

LC conditions

System: LC-MS/MS 4000 (Sciex)

Column oven CTO-20AC (Shimadzu)

Automatic sampler SIL20AC (Shimadzu)

Pump LC20AD (Shimadzu)

Pre-column: HPLC guard column with 4mm C18 cartridge

Column: AIT France Kromasil KR100 5C18, 50mm x 3.0mm, 5µm

Automatic ampler temperature: 4°C

Column temperature: 40°C

Flow: 1.0 mL/min

Mobile phase

Eluent A: Acetonitrile

Eluent B: Water containing 0.2% v/v acetic acid

Gradient:

Time (min)	% A	% B
0.0	10	90
1.0	50	50
2.5	50	50
3.0	90	10
3.5	90	10
4.0	10	90
5.0	10	90

Divert valve:

0.0 min to waste

0.8 min to MS

Injection volume: 10 µL

Retention time: Azoxystrobin, about 2.37 min

MS conditions

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Capillary voltage: 5500V

Temperature: 600°C

Scan type: MRM

Mass transitions (azoxystrobin): 404 → 372 (m/z) for quantification, 404 → 344 (m/z) for confirmation

Results and discussions

Table A 152160: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (µg/filter)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition <i>m/z</i> 404 → 372 <i>m/z</i> (Proposed for Quantification)							
Air	2.2	92, 93, 83, 67, 79	83	13	5	86	10
	22	93, 84, 95, 84, 94	90	6	5		
Transition <i>m/z</i> 404 → 344 <i>m/z</i> (Proposed for Confirmation)							
Air	2.2	92, 92, 81, 68, 80	83	12	5	86	10
	22	93, 83, 94, 82, 92	89	6	5		

Table A 153161: Characteristics for the analytical method used for validation of azoxystrobin in air

Azoxystrobin	
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). A reagent blank prepared according to the analytical method and the unfortified air filters showed no significant interference (< 30% LOQ at the retention time of azoxystrobin). The method is highly specific. Representative chromatograms for each analyte representing control samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent 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 (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A 154162 below.
Calibration range	Linearity was confirmed over the calibration range 0.00025 – 0.05 µg/mL (n = 8), which corresponds to no more than 30% of the LOQ at least + 20 % of the highest analyte concentration level.
Assessment of matrix effects is presented	Yes (not significant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 2.2 µg/filter for azoxystrobin in air (equivalent to 0.003 µg/m ³ air aspired). The LOD is considered as the lowest calibration standard used, 0.00025 µg/mL.
Stability of standards and extracts	The residues in final extracts are considered to be stable when store for 13 days between 0 and 9°C.

Table A 154162: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 125682893 x + 6495$, $r = 0.9988$ (n = 8)
404 → 344 m/z (Quantification)	$y = 22067150 x + 813$, $r = 0.9994$ (n = 8)

Conclusion

This analytical method for the determination of azoxystrobin content in air has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 2.2 µg/filter for azoxystrobin (equivalent to 0.003 µg/m³ air aspired).

A 2.1.2.5.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.5.3 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

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

A 2.1.2.6.1 Prothioconazole-desthio in bovine blood

A 2.1.2.6.1.1 Method validation

Comments of zRMS:	<p>The method has been evaluated and accepted by zRMS-PL in RR – Part B5 for CF-3307/ (January 2023). This method has not been reassessed in the framework of this application.</p> <p><u>Conclusion:</u></p> <p><i>The analytical method 01471 for the determination of prothioconazole-desthio in cattle blood by HPLC-MS/MS has been validated.</i></p> <p><i>Blood samples were diluted with acetonitrile and analyzed by HPLC-MS/MS using electrospray ionization in the positive mode.</i></p> <p><i>The limit of quantitation (LOQ) in blood samples for prothioconazole-desthio was 0.05 mg/L. Mean recoveries at all fortification levels (LOQ and 10-fold LOQ) were well within the 70–120% range. The relative standard deviations for the peak areas were ≤ 20% for all MRM transitions.</i></p> <p><i>The method meets all criteria of guidelines SANCO/825/00 rev. 8.1 to determine concentrations of prothioconazole-desthio in body fluid at the LOQ level of 0.05 mg/L, but according to the SANTE/2020/12830, Rev.2, 24. February 2021, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues.</i></p>
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Reference:	KCP 5.2/1517
Report	Validation of the BCS analytical method 01471 for the determination of prothioconazole-desthio in body fluid by HPLC-MS/MS, Hoeppe, S., 2015, Report N° M-535874-02-1
Guideline(s):	SANCO/3029/99; SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of this study was to validate the analytical enforcement method 01471 for the determination of prothioconazole-desthio in bovine blood (e.g. in case of intoxication) using HPLC-MS/MS. This method established an LOQ of 0.05 mg/L.

Prothioconazole-desthio is extracted and proteins are precipitated with acetonitrile. After centrifugation the supernatant is diluted with water and analysed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The triple-quadrupole is operated in the positive electrospray ionisation mode. Prothioconazole-desthio is monitored by means of the MS/MS transitions m/z 312 → 70 (quantitation) and m/z 312 → 125 (confirmation).

Analytical conditions

LC conditions

System: liquid chromatography with tandem mass spectrometric detection (LC-MS/MS)

MS conditions

Positive electrospray ionisation mode

Prothioconazole-desthio: 312 → 70 m/z proposed for quantification and 312 → 125 m/z proposed for confirmation.

Results and discussions

Table A 155163: Recovery results from method validation of prothioconazole-desthio using the analytical method

Prothioconazole-desthio				
Matrix	Fortification Level (µg/L)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 312 → 70 m/z (Proposed for Quantification)				
Cattle blood	50	85	4.2	5
	500	104	1.8	5
	Overall	94	11.0	10
Transition m/z 312 → 125 m/z (Proposed for Quantification)				
Cattle blood	50	91	7.1	5
	500	101	2.8	5
	Overall	96	7.7	10

Note : All the fortification levels are expressed as prothioconazole-desthio

Table A 156164: Characteristics for the analytical method used for validation of prothioconazole-desthio in bovine blood

	Prothioconazole-desthio
Specificity	Quantification by LC-MS/MS using at least two different MS/MS transitions ensures a high level of specificity. During the initial method validation, the apparent residues of in the control sample of blood were < 30% of LOQ. Untreated control samples were examined. All concentrations were below 30% of the LOQ (0.05 mg/L).
Calibration (type, number of data points)	The linearity of the method was demonstrated using matrix-matched standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$) performing with 1/x weighing. The correlation coefficients, r obtained were > 0.99
Calibration range	Linearity was confirmed over the calibration range 0.1 – 10.0 µg/L
Assessment of matrix effects is presented	Matrix-matched standards were used for quantification
Limit of determination/quantification	The limit of quantification for prothioconazole-desthio in blood was established at 50 µg/L, expressed as itself. The limit of detection (LOD) was estimated 10 µg/L.
Stability of standards and extracts	-

Conclusion

The method 01471 was developed for the determination of prothioconazole-desthio in blood. Quantification by means of LC-MS/MS with two MS/MS transitions ensures a high level of specificity. The results obtained during validation demonstrate accuracy and repeatability of the residue determination. The limit of quantification was established at 0.05 mg/L, expressed as prothioconazole-desthio.

A 2.1.2.6.1.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation. Full validation data were generated for two MS/MS transitions. The first transition is recommended for quantification and the second transition may

be used for confirmatory analyses.

A 2.1.2.6.1.3 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 2, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.2.6.2 Azoxystrobin and its isomer in human urine

A 2.1.2.6.2.1 Method validation

Comments of zRMS:	The analytical method has been validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin and R230310 in body fluids (human urine) with the LOQ of 0.05 mg/L. Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$. The method is acceptable. <u>Remark:</u> The limit of quantification was established at 0.05 mg/L, but according to the SANTE/2020/12830, Rev.2, 24. February 2021, the LOQ should be lower: 0.01 mg/L for body fluids.
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Reference:	KCP 5.2/4618
Report	Laboratory Validation of a method for the determination of Azoxystrobin and R230310 in body fluids, Sieckmann D., 2017, Report N°S17-01576
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Analytes were determined in human urine using a liquid chromatography technique (HPLC-MS/MS, 2 mass transitions monitored) after extraction with acetonitrile using QuEChERS method (4.0g of magnesium sulfate, 1.0g of sodium chloride, 1.0g of trisodium citrate dihydrate and 0.5g of disodium hydrogen citrate sesquihydrate). An aliquote was then cleaned-up by adding primary secondary amine (PSA) and C18 material. Before injection, samples were diluted twice (in LC water and in acetonitrile/water (1:9 v/v)) to be within the calibration range. The limit of quantification (LOQ) for azoxystrobin, and R230310 was 0.05 mg/L.

Analytical conditions

LC conditions

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

Pre-column: HPLC guard column with 4mm C18 cartridge

Column: MZ-Analysentechnik Kromasil 100-5 C18, 50 mm x 3.0 mm, 5 μ m

Column temperature: 40°C

Flow: 1.0 mL/min

Mobile phase

Eluent A: Water with 0.2% v/v acetic acid

Eluent B: Acetonitrile

Gradient:

Time (min)	% A	% B
0	10	90
1.0	50	50
2.5	50	50

3.0	90	10
3.5	90	10
4.0	10	90
5.0	10	90

Divert valve:

0.0 min to 1.0 min to waste

1.0 min to 3.5 min to MS

3.5 min to 5.1 min to waste

Injection volume: 20 µL

Retention time:

Azoxystrobin: About 2.3 min

R230310: About 1.9 min

MS conditions

MS system: SCIEX API 5500

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Acquisition mode: MS/MS, Multiple Reaction Monitoring (MRM)

Capillary voltage: 5500 V

Ionspray turbo heater: 400°C

Azoxystrobin and R230310: 404 → 372 m/z proposed for quantification and 404 → 344 m/z proposed for confirmation.

Results and discussions

Table A 157 165: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin					
Matrix	Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404 → 372 m/z (Proposed for Quantification)					
Human urine	0.05	104, 104, 100, 102, 99	102	2	5
Transition m/z 404 → 344 m/z (Proposed for Confirmation)					
Human urine	0.05	106, 104, 101, 102, 99	102	3	5

Recoveries are without any blank correction

Table A 1158 166: Recovery results from method validation of R230310 using the analytical method

R230310					
Matrix	Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates
Transition m/z 404 → 372 m/z (Proposed for Quantification)					
Human urine	0.05	108, 107, 103, 105, 101	105	3	5
Transition m/z 404 → 344 m/z (Proposed for Confirmation)					
Human urine	0.05	110, 107, 103, 105, 101	105	3	5

Recoveries are without any blank correction

Table A 159 167: Characteristics for the analytical method used for validation of azoxystrobin and R230310 residues in body fluids

Azoxystrobin and R230310	
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). A reagent blank and two control samples were extracted and analysed according to the analytical method. For both mass transitions, the samples showed no significant interference

	Azoxystrobin and R230310
	(above 30% of LOQ) at the retention times of the analytes in extracts of human urine. The method is highly specific. Representative chromatograms for each analyte representing control samples, samples fortified at the LOQ and also showing reagent blank extracts are provided (together with product ion spectra).
Calibration (type, number of data points)	The linearity of the method was demonstrated using solvent standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A16.8 below.
Calibration range	Linearity was confirmed over the calibration range 0.15 – 10.0 ng/mL ($n = 7$), corresponding to a range from 0.015 mg/L to 1.0 mg/L in sample extract which corresponds to more than 30% of the LOQ and at least + 20% of the highest analyte concentration detected in a sample extract.
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	The LOQ is defined as the lowest analyte concentration at which the methodology had been successfully validated. The LOQ is 0.05 mg/L for all analytes in human urine. The LOD is considered as the lowest calibration standard used and was set at 30% of the LOQ which is 0.015 mg/L for all analytes in human urine.
Stability of standards and extracts	Stock solutions prepared in acetonitrile are considered to be stable when stored for 18 days at 1°C to 10°C in the dark. Mix dilutions of stock solutions prepared in acetonitrile/water (1:1 v/v) are considered to be stable when stored for 18 days at 1°C to 10°C in the dark. Calibration solutions prepared in acetonitrile/water (1:9 v/v) are considered to be stable when stored for 9 days at 1°C to 10°C in the dark. The final extracts fortified at the LOQ level are considered to be stable when stored at 1 °C to 10 °C for 9 days in the dark.

Table A 16.168: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 3.89 \times 10^5 x + 2.2 \times 10^3$, $r = 1.0000$ ($n = 7$)
404 → 344 m/z (Confirmation)	$y = 1.17 \times 10^6 x + 1.1 \times 10^4$, $r = 1.0000$ ($n = 7$)

R230310	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 3.00 \times 10^5 x + 3.86 \times 10^3$, $r = 0.9999$ ($n = 7$)
404 → 344 m/z (Confirmation)	$y = 8.87 \times 10^5 x + 1.3 \times 10^4$, $r = 1.0000$ ($n = 7$)

Conclusion

This analytical method for the determination of azoxystrobin and R230310 content in human urine has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.05 mg/L for all analytes.

A 2.1.2.6.2.2 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.6.2.3 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.2.6.3 Azoxystrobin and its metabolite in human urine and plasma

Comments of zRMS:	<p>The analytical method has been validated according to the guidance document SANCO/825/00 rev. 8.1 for the determination of residues of azoxystrobin and its metabolite R234886 in human plasma and urine with the LOQ of 0.05 mg/L.</p> <p>Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$. The method is acceptable.</p> <p><u>Remark:</u></p> <p>The limit of quantification was established at 0.05 mg/L, but according to the SANTE/2020/12830, Rev.2, 24. February 2021, the LOQ should be lower: 0.01 mg/L for body fluids.</p>
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Reference:	KCP 5.2/ 17 19
Report	Validation of an analytical method for Azoxystrobin and its metabolite R234886 in human plasma and urine, Amic S., 2011, Report N°S11-02193
Guideline(s):	SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to validate an analytical method for the determination of azoxystrobin and its metabolite R234886 in human plasma and urine.

Analytes were determined in human plasma and urine using a liquid chromatography technique (HPLC-MS/MS, 2 mass transitions monitored) after extraction with acetonitrile/UP water (50/50 v/v) acidified with formic acid and dilution with acetonitrile /UP water (10/90 v/v). The limit of quantification (LOQ) for azoxystrobin, its metabolites R234886 was 0.05 µg/mL.

Analytical conditions

LC conditions

System: LC-MS/MS API 4000 (Sciex)

Pump LC20AD (Shimadzu)

Automatic sampler SILHTC (Shimadzu)

Column oven CTO-20AC (Shimadzu)

Column: AIT France Kromasil KR100 5C18, 50mm x 3.0mm, 5µm

Column temperature: 40°C

Automatic sampler temperature: 4°C

Flow: 1.0 mL/min

Mobile phase

Eluent A: Acetonitrile

Eluent B: 2% acetic acid v/v in ultra pure water

Gradient:

Time (min)	% A	% B
0	10	90
1.0	50	50
2.5	50	50
3.0	90	10
3.5	90	10
4.0	10	90
5.0	10	90

Divert valve:

From 0.0 min to 0.8 min to waste

From 0.8 min to MS

Injection volume: 10 and 100 µL

Retention time:

Azoxystrobin: About 2.6 min

R234886: About 1.9 min

MS conditions

Ionisation type: Electrospray ionisation (ESI)

Polarity: Positive ion mode

Acquisition mode: MRM

Azoxystrobin: 404 → 372 m/z proposed for quantification and 404 → 344 m/z proposed for confirmation.

R234886: 390 → 372 m/z proposed for quantification and 390 → 344 m/z proposed for confirmation.

Results and discussions

Table A 1261-169: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (µg/mL)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Human plasma	0.05	88, 90, 89, 83, 85	87	3	5	88	4
	0.5	94, 91, 91, 84, 86	89	5	5		
Human urine	0.05	92, 92, 96, 84, 90	91	5	5	94	6
	0.5	102, 101, 94, 92, 94	97	5	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Human plasma	0.05	85, 90, 91, 84, 86	87	3	5	88	4
	0.5	92, 90, 91, 83, 87	89	4	5		
Human urine	0.05	92, 92, 98, 85, 90	91	5	5	94	5
	0.5	101, 100, 93, 92, 94	96	4	5		

Table A 1362-170: Recovery results from method validation of R234886 using the analytical method

R234886							
Matrix	Fortification Level (µg/mL)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 390 → 372 m/z (Proposed for Quantification)							
Human plasma	0.05	86, 89, 89, 86, 86	87	2	5	89	3
	0.5	93, 93, 93, 87, 90	91	3	5		
	0.05	90, 88, 91, 86, 87	88	2	5	89	3
	0.5	92, 92, 91, 86, 87	90	3	5		
Transition m/z 390 → 344 m/z (Proposed for Confirmation)							
Human plasma	0.05	90, 92, 89, 92, 95	91	3	5	91	3
	0.5	92, 93, 93, 87, 86	90	4	5		
Human urine	0.05	90, 94, 90, 88, 85	89	4	5	89	4
	0.5	89, 92, 93, 85, 87	89	4	5		

Table A 163-171: Characteristics for the analytical method used for validation of azoxystrobin and R234886 residues in human plasma and urines

	Azoxystrobin and R234886
Specificity	MS/MS determination was conducted by monitoring two mass transitions (azoxystrobin: 404 → 372 m/z for quantification and 404 → 344 m/z for confirmation, R234886: 390 → 372 m/z for quantification and 390 → 344 m/z for confirmation). A reagent blank prepared according to the analytical method and the unfortified human plasma and urine specimens showed no significant interference (< 30% LOQ) at the retention time of azoxystrobin and its metabolites R234886. The method is highly specific. Representative chromatograms for each matrix and analyte representing control samples, samples fortified at the LOQ and samples fortified at 10 x LOQ and showing reagent blank

	Azoxystrobin and R234886
	extracts are provided.
Calibration (type, number of data points)	The linearity of the method was demonstrated using non-matrix matched calibration standards. Linear calibration functions were calculated by regression analysis (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99 . Please see table A164 below.
Calibration range	Linearity was confirmed over the calibration range 0.00025 – 0.05 µg/mL ($n = 7$ or 8), corresponding to a range from 0.0125 µg/mL to 2.5 µg/mL in sample extract which corresponds to more than 30% of the LOQ to 500 % above the highest level of recovery validated.
Assessment of matrix effects is presented	Yes
Limit of determination/quantification	The LOQ is defined as the lowest sample for which acceptable recovery and repeatability were demonstrated. The LOQ is 0.05 µg/mL for all analytes in human plasma and urine. The LOD is considered as the lowest calibration standard used (0.00025 µg/mL), equivalent to 0.0125 µg/mL for all analytes in human plasma and urine.
Stability of standards and extracts	The residues in final extract were proved to be stable for 11 days between 0 and 9°C.

Table A 164172: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 284435 x + 30841$, $r = 0.9964$ ($n = 8$)
404 → 344 m/z (Confirmation)	$y = 30299 x + 2497$, $r = 0.9990$ ($n = 7$)

R234886	
Transition	Linearity data
390 → 372 m/z (Quantification)	$y = 45207 x + 1751$, $r = 0.9999$ ($n = 8$)
390 → 344 m/z (Confirmation)	$y = 6885 x + 131$, $r = 0.9998$ ($n = 8$)

Conclusion

This analytical method for the determination of azoxystrobin and its metabolites R234886 content in human plasma and urine has been acceptably validated for specificity, linearity, accuracy and precision of the method and SANCO/825/00 rev.8.1 requirements were fulfilled. In addition, SANTE/2020/12830 rev.1 requirements were also fulfilled. The Limit of Quantification was 0.05 µg/mL for all analytes.

A 2.1.2.6.3.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.6.3.2 Extraction efficiency

According to SANTE 2017/10632 Rev. 4 23 February 2022, 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.

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

A 2.1.2.6.4 Azoxystrobin and its metabolite in body fluids

Comments of zRMS:	The multi-residue method QuEChERS has been successfully validated according to the guidance documents SANTE/2020/12830 rev.2 for the determination of residues of azoxystrobin in body fluids (whole blood) with the LOQ of 0.01 mg/L. Mean recoveries were in the range of 70 – 110% with relative standard deviations of $\leq 20\%$. The method is acceptable.
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Reference:	KCP 5.2/1820
Report	Azoxystrobin – Azoxystrobin (ICI5504): Validation of Analytical QuEChERS Method for the Determination of Residues of Azoxystrobin in Body Fluid by LC-MS/MS, Harper H., 2022, Report No. 8485925
Guideline(s):	SANTE/2020/012830 rev1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and method

The objective of the study was to validate a QuEChERS analytical method for the determination of residues of azoxystrobin in body fluids (whole blood).

2 mL of matrix were transferred into a 50mL polypropylene tube. 8 mL of water and 10 mL of acetonitrile were added and the tube was shaken vigorously for approximately 1 minute. The content of the citrate extraction tube was added and the tube was shaken vigorously for approximately 1 minute. The tube was centrifuged at 3500 rpm for 5 minutes. 1 mL of the upper acetonitrile phase was transferred into a QuEChERS Dispersive tube and was shaken vigorously by hand for approximately 1 minute. The sample was then centrifuged at 3500 rpm for 5 minutes. 400 μ L of the upper acetonitrile layer was transferred into a 15 mL polypropylene tube and completed up to 2 mL with water. The final extract was analysed for azoxystrobin using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantification (LOQ) for azoxystrobin was 0.01 mg/L.

Analytical conditions

LC conditions

System: Waters Acquity UPLC System

Column: Acquity UPLC BEH C₁₈, 50mm x 2.1mm, 1.7 μ m

Column temperature: 40°C

Injection volume: 10 μ L

Flow: 0.5 mL/min

Mobile phase

Eluent A: 0.2% acetic acid in water

Eluent B: Acetonitrile

Gradient:

Time (min)	% A	% B
0.0	90	10
1.0	50	50
2.5	50	50
3.0	10	90
3.5	10	90
4.0	90	10
5.0	90	10

Retention time:

Azoxystrobin: About 1.8 min

MS conditions

MS system : API 4000 Mass spectrometer, Applied Biosystems

Ionisation type: Ion spray

Polarity: Positive ion mode

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

Azoxystrobin: 404 → 372 m/z used for quantification and 404 → 344 m/z used for confirmation.

Results and discussions

Table A 165 173: Recovery results from method validation of azoxystrobin using the analytical method

Azoxystrobin							
Matrix	Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Transition m/z 404 → 372 m/z (Proposed for Quantification)							
Whole blood	0.01	108, 98, 102, 105, 99	102	4.1	5	99	5.0
	0.1	95, 93, 97, 96, 94	95	1.7	5		
Transition m/z 404 → 344 m/z (Proposed for Confirmation)							
Whole blood	0.01	109, 103, 102, 105, 101	104	3.0	5	100	5
	0.1	97, 96, 97, 97, 92	96	2.3	5		

Table A 166 174: Characteristics for the analytical method used for validation of azoxystrobin in whole blood

	Azoxystrobin
Specificity	LC-MS/MS determination was conducted by monitoring two mass transitions (404 → 372 m/z for quantification and 404 → 344 m/z for confirmation). The method is considered highly specific. No residues of azoxystrobin were detected above 30% of the LOQ in any of the control and reagent blank samples, indicating that no interferences were present at the retention time of azoxystrobin in the whole blood. Representative chromatograms representing control and blank samples, the lowest calibration level, samples fortified at the LOQ and samples fortified at 10 x LOQ, blank reagent are provided. Azoxystrobin mass spectrum is also 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 performed with 1/x weighing (regression model: $y = a \cdot x + b$). The correlation coefficients, r obtained were > 0.99. Please see table A167 below.
Calibration range	Linearity was confirmed over the calibration range 0.1 – 5.0 ng/mL (n = 7), corresponding to a range from 0.0025 – 0.125 mg/L in matrix which corresponds to 25% of the LOQ to at least 20 % above the highest analyte concentration in the final sample extracts.
Assessment of matrix effects is presented	Yes (insignificant)
Limit of determination/quantification	The LOQ is defined as the lowest validated level where acceptable recovery and repeatability were demonstrated. The LOQ is 0.01 mg/L for azoxystrobin in whole blood. The LOD is considered as the lowest calibration standard used, 0.0025 mg/L (25% of the LOQ).
Stability of standards and extracts	The residues in final extracts (10 x LOQ) are considered to be stable when stored for 10 days between 2 and 8°C. Standard solutions prepared in acetonitrile/water (50/50 v/v) are considered to be stable when stored 208 days between 2 and 8°C.

Table A 168 175: Linearity of detector response

Azoxystrobin	
Transition	Linearity data
404 → 372 m/z (Quantification)	$y = 130766 x + 6697.05$, $r = 0.9983$ (n = 7)
404 → 344 m/z (Quantification)	$y = 31984.4 x + 1197.32$, $r = 0.9993$ (n = 7)

Conclusion

This analytical method for the determination of azoxystrobin content in body fluids (whole blood) has been

acceptably validated for specificity, linearity, accuracy and precision of the method and SANTE/2020/12830 rev.1 requirements were fulfilled. The Limit of Quantification was 0.01 mg/L for azoxystrobin.

A 2.1.2.6.4.1 Confirmatory method (if required)

Confirmatory data presented in initial method validation.

A 2.1.2.6.4.2 Extraction efficiency

As stated in SANTE/2020/12830 Rev. 1, extraction efficiency should be addressed in methods for the determination of residues in food/feed of plant and animal origin only. Consequently, extraction efficiency is not relevant for this analytical method.

A 2.1.2.7 Other Studies/ Information

No new or additional studies have been submitted.