

FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: CHR/F/SCORPIO 325 SC

Product name(s): Clapton 325 SC, Scarp 325 SC

Chemical active substance(s):

Difenoconazole, 125 g/L

Azoxystrobin, 200 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: Innvigo Sp. z o.o.

Submission date: 01.2024

Finalisation date: 11/2025

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This document describes the acceptable use conditions required for registration according to Art. 34 and Art. 33 of Regulation 1107/2009 of CHR/F/SCORPIO 325 SC containing azoxystrobin and difenoconazole in POLAND.

Scorpion 325 SC is the original product to which Innvigo Sp. z o.o. would like to refer. 10 years for registration data of Scorpion 325 SC was expired in Poland. Thus, the data protection of studies provided in registration report of Scorpion 325 SC has expired. Innvigo Sp. z o.o. refers to above mentioned studies within this document.

zRMS comments:

The text highlighted in grey was provided by the zRMS.

Scorpion 325 SC was authorized in Poland in 2013 (authorization number R-6/2013 wu). The authorization was valid until 31/12/2025. It should be noted that the label for Scorpion 325 SC does not cover use on oilseeds crops and sugar beets which are proposed in the GAP for CHR/F/SCORPIO 325 SC.

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Version history

When	What
07/2025	Draft registration report provided by zRMS for commenting
11/2025	Final RR

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5 Analytical methods

5.1 Conclusion and summary of assessment

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

Noticed data gaps are: None

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

Noticed data gaps are: None

Commodity/crop	Supported/ Not supported
Winter oilseed rape	Supported
Sugar beet	Supported
Spring oilseed rape	Supported
~~Sinapis alba (White mustard)~~ Mustard	Supported
Papaver somniferum (Breadseed poppy)	Supported
~~Glycine max~~ Soybean	Supported
~~Helianthus annuus~~ Sunflower	Supported
~~Cucurbita pepo~~ Pumpkin seed	Supported
~~Linum usitatissimum~~ Flax	Supported
~~Cannabis sativa~~ Hemp	Supported
~~Brassica napus subsp. Rapifera (Swede)~~ Turnip	Supported
Cichorium intybus ~~(Common chicory)~~ root	Supported
~~Beta vulgaris subsp. Vulgaris (Leaf beet)~~ Beetroot	Supported
~~Rutabaga~~	Supported
~~Armoracia rusticana~~ Horseradish	Supported
~~Apium graveolens var. rapaceum~~ Celeriac (root)	Supported
Ornamental nursery plants	Not required
Forest nursery plants	Not required
Renewals, afforestation and plantations of forest trees	Not required
Salix viminalis	Not required
Wicker	Not required
~~Nicotiana tabacum (Tobacco)~~ Tobacco	Not required

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

Comments of zRMS:	Accepted
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Reference:	KCP 5.1/01
Report	Validation of analytical method for CHR/F/SCORPIO 325 SC for determination of azoxystrobin, difenoconazole and impurities Z-azoxystrobin and toluene, I.Knapik, Study code: ICB/52/2023
Guideline(s):	
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

- acetonitrile (Merck),
- 85% phosphoric acid (VWR),
- azoxystrobin standard; Sigma Aldrich; batch BCCF6593, prod. date: March 25, 2021; exp. date: February 2026; storage conditions: 4±2°C,
- difenoconazole standard; Sigma Aldrich; batch BCCD4900, prod. date: August 17, 2020; exp. date: June, 2025; storage conditions: 4±2°C,
- standard stock solution of azoxystrobin in acetonitrile (for calibration) (Table 4),
- standard stock solution of difenoconazole in acetonitrile (for calibration) (Table 5),
- working standard solutions of azoxystrobin and difenoconazole in acetonitrile (for calibration) (Table 6),
- standard stock solution of azoxystrobin in acetonitrile (for recovery) (Table 7),
- standard stock solution of difenoconazole in acetonitrile (for recovery) (Table 8),
- analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16,
- ultrasonic bath, W/29 (Branson, Taiwan),
- liquid chromatograph with diode array detection (Shimadzu, Japan), WP/42, WP/19,
- chromatography columns type C18, 100Å, 150 mm x 4,6 mm, 5 µm; Luna Omega Polar, Phenomenex, K/15/HPLC,
- chromatography column type C18, 250 mm x 4,6 mm, 5 µm (Zorbax Eclipse Plus, Agilent), K/11/HPLC,
- chromatographic vials 1.5 mL with septa buthyl/Teflon,
- volumetric flasks A class 10 mL,
- measuring syringes 10 µL, 100 µL, 250 µL, 500 µL, 1000 µL.

Validation was carried out according to Standard Operational Procedure SPB/179. Content of azoxystrobin at a level of 200 g/L and difenoconazole at a level of 125 g/L in the test item was determined accordingly by liquid chromatography with diode array detection (HPLC-DAD).

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Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances azoxystrobin and difenoconazole in plant protection product CHR/F/SCORPIO 325 SC:

	Azoxystrobin	Difenoconazole																																																																																
Author(s), year	Knapik I., 2023																																																																																	
Principle of method	HPLC-DAD																																																																																	
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r) n=5	In order to check the linearity of azoxystrobin, calibration curve was prepared using standard solutions. A graph of the peak area to the concentration of azoxystrobin was plotted. The resulting curve is linear in the tested concentrations. Linearity range of azoxystrobin is - 1.039 to 380.10 µg/mL. Correlation coefficient R ² is s 0.9999938 (for primary chromatographic system. Correlation coefficient R ² is 0.9999927 for secondary chromatographic system.	In order to check the linearity of difenoconazole, calibration curve was prepared using standard solutions. A graph of the peak area to the concentration of difenoconazole was plotted. The resulting curve is linear in the tested concentrations. Linearity range of difenoconazole is 1.050 to 307.04 µg/mL. Correlation coefficient R ² is 0.9999295 for primary chromatographic system. Correlation coefficient R ² is 0.9998947 for secondary chromatographic system.																																																																																
Precision – Repeatability Mean n=5 (%RSD)	According to SPB/179, the parameters obtained as a result of validation should meet the following criteria: - precision for azoxystrobin ≤ 1.73 (validation level 100%) - precision for azoxystrobin ≤ 3.11 (validation level LOQ) - precision for azoxystrobin ≤ 1.81 (validation level ULOQ) - Horwitz ratio ≤ 1.0.	According to SPB/179, the parameters obtained as a result of validation should meet the following criteria: - precision for difenoconazole ≤ 1.86 (validation level 100%) - precision for difenoconazole ≤ 3.11 (validation level LOQ) - precision for difenoconazole ≤ 1.87 (validation level ULOQ) - Horwitz ratio ≤ 1.0																																																																																
Accuracy n=5 (% Recovery)	Primary chromatographic system <table><tr><th>Validation level</th><th>Active ingredient</th><th>Precision [%]</th><th>Horwitz ratio</th></tr><tr><td>100% without standard addition</td><td>azoxystrobin</td><td>0.14</td><td>0.08</td></tr><tr><td>100% with standard addition (20-30%)</td><td>azoxystrobin</td><td>-</td><td>-</td></tr><tr><td>LOQ</td><td>azoxystrobin</td><td>1.80</td><td>0.58</td></tr><tr><td>ULOQ</td><td>azoxystrobin</td><td>1.06</td><td>0.59</td></tr></table> Secondary chromatographic system <table><tr><th>Validation level</th><th>Active ingredient</th><th>Precision [%]</th><th>Horwitz ratio</th></tr><tr><td>100% without standard addition</td><td>azoxystrobin</td><td>0.46</td><td>0.27</td></tr><tr><td>100% with standard addition (20-30%)</td><td>azoxystrobin</td><td>-</td><td>-</td></tr><tr><td>LOQ</td><td>azoxystrobin</td><td>2.64</td><td>0.85</td></tr><tr><td>ULOQ</td><td>azoxystrobin</td><td>0.95</td><td>0.52</td></tr></table>	Validation level	Active ingredient	Precision [%]	Horwitz ratio	100% without standard addition	azoxystrobin	0.14	0.08	100% with standard addition (20-30%)	azoxystrobin	-	-	LOQ	azoxystrobin	1.80	0.58	ULOQ	azoxystrobin	1.06	0.59	Validation level	Active ingredient	Precision [%]	Horwitz ratio	100% without standard addition	azoxystrobin	0.46	0.27	100% with standard addition (20-30%)	azoxystrobin	-	-	LOQ	azoxystrobin	2.64	0.85	ULOQ	azoxystrobin	0.95	0.52	Primary chromatographic system <table><tr><th>Validation level</th><th>Active ingredient</th><th>Precision [%]</th><th>Horwitz ratio</th></tr><tr><td>100% without standard addition</td><td>azoxystrobin</td><td>0.14</td><td>0.08</td></tr><tr><td>100% with standard addition (20-30%)</td><td>azoxystrobin</td><td>-</td><td>-</td></tr><tr><td>LOQ</td><td>azoxystrobin</td><td>1.80</td><td>0.58</td></tr><tr><td>ULOQ</td><td>azoxystrobin</td><td>1.06</td><td>0.59</td></tr></table> Secondary chromatographic system <table><tr><th>Validation level</th><th>Active ingredient</th><th>Precision [%]</th><th>Horwitz ratio</th></tr><tr><td>100% without standard addition</td><td>difenoconazole</td><td>0.24</td><td>0.13</td></tr><tr><td>100% with standard addition (20-30%)</td><td>difenoconazole</td><td>-</td><td>-</td></tr><tr><td>LOQ</td><td>difenoconazole</td><td>0.85</td><td>0.27</td></tr><tr><td>ULOQ</td><td>difenoconazole</td><td>0.31</td><td>0.17</td></tr></table>	Validation level	Active ingredient	Precision [%]	Horwitz ratio	100% without standard addition	azoxystrobin	0.14	0.08	100% with standard addition (20-30%)	azoxystrobin	-	-	LOQ	azoxystrobin	1.80	0.58	ULOQ	azoxystrobin	1.06	0.59	Validation level	Active ingredient	Precision [%]	Horwitz ratio	100% without standard addition	difenoconazole	0.24	0.13	100% with standard addition (20-30%)	difenoconazole	-	-	LOQ	difenoconazole	0.85	0.27	ULOQ	difenoconazole	0.31	0.17
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LOQ	difenoconazole	0.85	0.27																																																																															
ULOQ	difenoconazole	0.31	0.17																																																																															
Interference/ Specificity	Specificity of the method was evaluated based on the analysis of samples chromatograms against chromatograms of standards azoxystrobin, difenoconazole and peaks purity. Analysis showed no overlapping of determined ingredients signals with the signals																																																																																	

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	Azoxystrobin	Difenoconazole
	of matrix components under method conditions, hence method specificity criterion is fulfilled.	

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

Comments of zRMS: Accepted

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

Reference:	KCP 5.1.1/02
Report	Validation of analytical method for CHR/F/SCORPIO 325 SC for determination of azoxystrobin, difenoconazole and impurities Z-azoxystrobin and toluene
Guideline(s):	
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods:

Azoxystrobin

- acetonitrile (Merck),
- 85% phosphoric acid (VWR),
- Z-azoxystrobin standard; TRC; batch 1-KYM-40-2, prod. date: August 31, 2021; exp. date: August 29, 2025; storage conditions: 4±2°C,
- Z-azoxystrobin standard stock solution in acetonitrile (for calibration) (Table 9),
- working standard solutions of Z-azoxystrobin in acetonitrile (for calibration) (Table 10),
- working standard solution of Z-azoxystrobin in acetonitrile (for recovery) (Table 11),
- analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16,
- ultrasonic bath, W/29 (Branson, Taiwan),
- syringe filter with a pore diameter of 0.45 µm,
- liquid chromatographs with diode array detection (Shimadzu, Japan), WP/42, WP/19,
- chromatography columns type C18, 100Å, 150 mm x 4,6 mm, 5 µm; Luna Omega Polar, Phenomenex, K/15/HPLC,
- chromatography column type C18, 250 mm x 4,6 mm, 5 µm (Zorbax Eclipse Plus, Agilent), K/11/HPLC,
- chromatographic vials 1,5 mL with septa buthyl/teflon,
- volumetric flasks class A, 10 mL,
- measuring syringes 10 µL, 100 µL, 250 µL, 500 µl and 1000 µL. - liquid chromatographs with diode array detection (Shimadzu, Japan), WP/42, WP/19,
- chromatography columns type C18, 100Å, 150 mm x 4,6 mm, 5 µm; Luna Omega Polar, Phenomenex, K/15/HPLC,
- chromatography column type C18, 250 mm x 4,6 mm, 5 µm (Zorbax Eclipse Plus, Agilent), K/11/HPLC,
- chromatographic vials 1,5 mL with septa buthyl/teflon,

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- volumetric flasks class A, 10 mL,
- measuring syringes 10 µL, 100 µL, 250 µL, 500 µl and 1000 µL.

Difenoconazole:

- acetone GC (VWR),
- analytical standard of toluene, batch BCCJ0157 (Sigma-Aldrich), prod. date: July 28, 2022; exp. date: July, 2025; storage conditions: 4±2°C,
- analytical standard of fluorobenzene (internal standard - IS), batch BCCH0723 (SigmaAldrich), prod. date: January 19, 2022; exp. date: December, 2024; storage conditions: 4±2°C,
- standard stock solution of toluene in acetone (for calibration) (Table 12),
- standard solution of fluorobenzene (IS) in acetone (Table 13),
- working standard solutions of toluene in acetone (for calibration) (Table 14),
- standard working solution of toluene in acetone (for recovery) (Table 15),
- analytical balance – accuracy 0.0001 g, WP/16 (Ohaus, Switzerland),
- ultrasonic bath, W/29 (Branson, Taiwan),
- syringe filter with a pore diameter of 0.45 µm,
- gas chromatograph Shimadzu GC2010 with mass detection (GC-MS), WP/38 (Shimadzu, Japan),
- chromatography column ZB-624 30 m, I.D. = 0.25 mm, df = 1.4 µm, K/4/GC (Phenomenex),
- chromatographic vials 1.5 ml with septa buthyl/Teflon,
- volumetric flasks A class 10 mL,
- measuring syringes 10, 50, 100, 250, 500, 1000 µL.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) CHR/F/SCORPIO 325 SC SC

	Z-azoxystrobin	Toluene
Author(s), year	I.Knapik, 2023	
Principle of method	HPLC-DAD	GC-MS
Linearity (linear between mg/L) (correlation coefficient, expressed as r) n=5	In order to check the linearity of Z-azoxystrobin, calibration curve was prepared using standard solutions with concentrations A graph of the peak area to the concentration of Z-azoxystrobin was plotted. The resulting curve is linear in the tested concentrations. Linearity range of Z-azoxystrobin is from 1.0868 to 98.800 µg/mL. Correlation coefficient R2 is 0.9999809 primary chromatographic system. Correlation coefficient	In order to check the linearity of toluene, calibration curve was prepared using standard solutions with concentrations A graph of the ratio of peak area of toluene to the area internal standard was plotted against the ratio of toluene concentration to the internal standard concentration. The resulting curve is linear in the tested concentrations. Linearity range of toluene is from 2.700 to 32.400 µg/mL. Correlation coefficient R2 is 0.999649

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	Z-azoxystrobin	Toluene
	R2 is 0.9999562 for secondary chromatographic system.	
Precision – Repeatability Mean n=5 (%RSD)	According to SPB/179, the parameters obtained as a result of validation should meet the following criteria: - linearity $R^2 \geq 0.99$ - Horwitz ratio ≤ 1.0	According to SPB/179, the parameters obtained as a result of validation should meet the following criteria: - linearity $R^2 \geq 0.99$, - Horwitz ratio ≤ 1.0
Accuracy n=5 (% Recovery)	0.0001 g	0.0001 g
Interference/ Specificity	Specificity of the method was evaluated based on the analysis of chromatograms for samples against chromatogram of Z-azoxystrobin standard. Analysis showed no overlapping of determined impurity signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.	Specificity of the method was evaluated based on the analysis of chromatograms for samples against chromatograms of standard toluene. Specificity and selectivity of validated method for toluene determination was assessed at the point of optimizing conditions of analysis, by obtaining parameters for the best impurity separation while maintaining interference impact at its lowest. Toluene was identified by the presence of specific fragmentation ions and determined by the target ions. The method was set up in the way the percentage ratio between value of the main ion to identified reference ions doesn't exceed 30% value of the error from the mass spectrum. Ions have been selected by NIST 11 library.
LOQ	~~0.83~~ 1.0868 µg/ml	~~3.55~~ 2.700 µg/ml
Comment	The validation parameters are within the acceptance range and fulfil EU requirements.	

Conclusion

It was confirmed that the method is specific. There were no peaks from placebo interfering with determined compounds. The validation parameters (specificity, linearity, instrument precision, repeatability and accuracy) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev.4.

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

Please refer to PART C – Confidential data.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1) product.

Analytical methods for determination of difenoconazole and azoxystrobin, impurities and relevance of CIPAC methods in CHR/F/SCORPIO 325 SC were not evaluated as part of the EU review. Therefore, all relevant data are provided and are considered adequate.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of azoxystrobin and difenoconazole for the generation of pre-authorization data is given in the following table for the detailed evaluation of additional studies it is referred to Appendix 1.

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

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 (Residues)	Primary Azoxystrobin and R230310 (Z-isomer) LC-MS/MS LOQs 0.01 mg/kg (cabbage, cereals (grain and straw) for each ILV (LOQ 0.01 mg/kg, cabbage, kale, potato, maize, lettuce, sugar beet) DFG S19 multi-method is applicable to cereals and other dry crops" (grain and straw), „fruits with high acid content" (grapes) and commodities with high water content (wine) LOQs: 0.02 mg/kg (straw); 0.01 mg/kg (grain, grapes); 10 µg/l (wine) DFG S19 for plant	0.01 mg/kg	LC-MS/MS	Robinson, N.J. et al, 1999, Lister, N., 1999, B.5.2.1.2, DAR, Azoxystrobin – Methods of analysis EU agreed EFSA Journal 2010;8(4):1542

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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
	matrices of high water content appropriate for brassica vegetable commodities is not available.			
	Primary (Oilseed rape: seed, plant, straw)	0.010 mg/kg	LC/MS/MS	Niewielt-Stasiak S., 2023, VAL/22/2023 Not presented on EU level
	Primary (Sugar beet: roots, leaves)	0.010 mg/kg	LC-MS/MS	Niewielt-Stasiak S., 2023, VAL/22/2023 Not presented on EU level
	Confirmatory (if required)	N/A	N/A	
Animal products, food of animal origin (Residues)	Primary Azoxystrobin and R230310 (Z-isomer)	0.01 mg/kg (liver, muscle, fat, eggs) for each compound, 0.001 mg/kg milk	GC-NPD	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis EU agreed EFSA Journal 2010;8(4):1542
	Primary (honey)	0.010 mg/kg	LC-MS/MS	Mahlow S., Ezeobi-Chiris C., 2023, S22-08436 Not presented on EU level
	Confirmatory (if required)	N/A	N/A	
Soil	Primary	0.02 mg/kg (0.01 mg/kg for R401553, R402173)	HPLC-MS/MS HPLC-UV and GC-MSD	Johnson, R.I., et al., 2000, B.5.3.1.1, DAR, Azoxystrobin – Methods of analysis EU agreed EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	N/A	N/A	
Water	Primary	0.01 µg/mL	GC-MSD	Robinson, N.J., 2000, B.5.3.2.1, DAR, Azoxystrobin – Methods of analysis EU agreed EFSA Journal 2010;8(4):1542

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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 (if required)	N/A	N/A	
Human blood	Primary	0.01 µg/mL	LC-MS/MS	Hall, M.G., 1999, IIIA 5.8, Registration Report Part B, Section 2: Analytical Methods, Syngenta, 16 May 2013
	Confirmatory (if required)	N/A	N/A	
Air	Primary	3 µg/m ³	GC-MSD	Crawford, N., 2001, B.5.3.3.1, DAR, Azoxystrobin – Method of analysis EU agreed EFSA Journal 2010;8(4):1542
	Confirmatory (if required)	N/A	N/A	

Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants (Residues)	Primary	0.02 mg/kg (high water) 0.05 mg/kg (oily, dry) 0.02 mg/kg (apple, lettuce), 0.05 mg/kg (wheat grain, oilseed rape) ILV available down to 0.01 mg/kg for all matrices	LC-MS/MS	Steinhauer S. (2004a), B.5.2.1, DAR, Difenoconazole – Method of analysis EU agreed EFSA Journal 2011;9(1):1967
	Primary (Oilseed rape: seed, plant, straw)	0.010 mg/kg	LC/MS/MS	Niewielt-Stasiak S., 2023, VAL/22/2023 Not presented on EU level
	Primary (Sugar	0.010 mg/kg	LC-MS/MS	Niewielt-Stasiak S., 2023, VAL/22/2023

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Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	beet: roots, leaves)			Not presented on EU level
	Confirmatory (if required)	N/A	N/A	
Animal products, food of animal origin (Residues)	Primary Difenoconazole and CGA 205375	0.01 mg/kg (tissues, fat, eggs), 0.005 mg/kg (milk) ILV available down to LOQ	LC-MS/MS	Crook S.J (2004), B.5.2.2, DAR, Difenoconazole – Method of analysis Ryan J (2004b), B.5.2.2, DAR, Difenoconazole – Method of analysis EU agreed EFSA Journal 2011;9(1):1967
	Primary (honey)	0.010 mg/kg	LC-MS/MS	Mahlow S., Ezeobi-Chris C., 2023, S22- 08436 Not presented on EU level
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Benaseraf L. (2004), B.5.2.2, DAR – Method of analysis
Soil	Primary Difenoconazole and CGA 205375	0.01 mg/kg	LC-MS/MS	Tummon O.J (2004a), B.5.3.1, DAR, Difenoconazole – Method of analysis EU agreed EFSA Journal 2011;9(1):1967
	Confirmatory (if required)	N/A	N/A	
Water	Primary	0.05 µ/L (potable water), 0.1 µ/L (surface water)	GC/ECD	Tribolet R. (1999a), B.5.3.2, DAR, Difenoconazole – Method of analysis Tribolet R. (1999b), B.5.3.2, DAR, Difenoconazole – Method of analysis EU agreed EFSA Journal 2011;9(1):1967
	Confirmatory (if required)	N/A	N/A	
Feed, body fluids (Toxicology)	Primary	N/A	Analytical method is not required since difenoconazole is not classified as toxic or highly toxic.	
	Confirmatory (if required)	N/A	Analytical method is not required since difenoconazole is not classified as	

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Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
			~~toxic or highly toxic.~~	
Air	Primary	0.99 ng/L	LC-MS/MS	Tummon O.J (2004b), B.5.3.4, DAR, Difenoconazole - Method of analysis EU agreed EFSA Journal 2011;9(1):1967
	Confirmatory (if required)	N/A	N/A	

Component of residue definition: Triazole Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plant (Residues)	Primary (Oilseed rape: seed, plant, straw residues)	0.010 mg/kg	LC/MS/MS	Niewielt-Stasiak S., 2022, VAL/10/2021 Not presented on EU level
	Primary (Sugar beet: roots, leaves)	0.010 mg/kg	LC-MS/MS	Niewielt-Stasiak S., 2022, VAL/21/2023 Not presented on EU level
	Confirmatory (if required)	Same as primary.		

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

All data is described in EU approved documents for :

- DAR, Azoxystrobin – Volume 3, Annex B.5: Methods of analysis (2009)
- DAR, Difenoconazole – Volume 3, Annex B.5: Analytical methods (2006)

5.3.1 Analysis of the plant protection product (KCP 5.2)

For active substances azoxystrobin and difenoconazole all presented methods are sufficient and no new methods are necessary. Please refer to KCP 5.1.2.

5.3.2 Description of analytical methods for the determination of residues

azoxystrobin. (KCP 5.2)

Please refer to Appendix 1.

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

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

Table 5.3-13: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Azoxystrobin	0.01 mg/kg	Reg. (EU) No ~~2023/129~~ 2024/2633
Plant, high acid content	Azoxystrobin	0.01 mg/kg	Reg. (EU) No ~~2023/129~~ 2024/2633
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	Reg. (EU) No ~~2023/129~~ 2024/2633
Plant, high oil content	Azoxystrobin	0.01 mg/kg	Reg. (EU) No ~~2023/129~~ 2024/2633
Plant, difficult matrices (hops)	Azoxystrobin	0.05 mg/kg, (30 mg/kg for hops)	Reg. (EU) No ~~2023/129~~ 2024/2633
Muscle	Azoxystrobin	0.01 mg/kg	Reg. (EU) No ~~2023/129~~ 2024/2633
Milk		0.01 mg/kg	
Eggs		0.01 mg/kg	
Fat		0.01 mg/kg	
Liver		0.01 mg/kg	
Soil	Azoxystrobin	0.05 mg/kg	common limit
Drinking water	Azoxystrobin	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Azoxystrobin	9.54 µg/L	NOEC <i>Mysidopsis bahia</i> , EFSA Journal 2010; 8(4):1542
Air	Azoxystrobin	~~3~~ 60 µg/m ³	AOEL 0.2 mg/kg bw/day EFSA Journal 2010; 8(4):1542
Tissue (meat or liver)	Azoxystrobin	0.01 mg/kg	~~Reg. (EU) No 2023/129~~ SANTE/2020/12830, Rev.2
Body fluids	Azoxystrobin	0.01 mg/ ~~kg~~ L	~~Reg. (EU) No 2023/129~~ SANTE/2020/12830, Rev.2

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

An overview on the acceptable methods and possible data gaps for analysis of azoxystrobin in plant

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matrices is given in the following tables. ~~For the detailed evaluation of additional studies it is referred to Appendix 1.~~

Table 5.3-24: 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	LC-MS/MS	Robinson, N.J. et al, 1999, B.5.2.1.2, DAR, Azoxystrobin – Methods of analysis Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
	ILV	0.01 mg/kg	LC-MS/MS	Kang, J., 2003, B.5.2.1.3, DAR, Azoxystrobin – Methods of analysis
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
High acid content	Primary	0.01 mg/kg	LC-MS/MS	Robinson, N.J. et al, 1999, B.5.2.1.2, DAR, Azoxystrobin – Methods of analysis Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
	ILV	N/A	N/A	
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
High oil content	Primary	0.01 mg/kg	LC-MS/MS	Robinson, N.J. et al, 1999, B.5.2.1.2, DAR, Azoxystrobin – Methods of analysis Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
	ILV	N/A	N/A	
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
High protein/high starch content (dry)	Primary	0.01 mg/kg	LC-MS/MS	Robinson, N.J. et al, 1999, B.5.2.1.2, DAR, Azoxystrobin – Methods of analysis Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis
	ILV	0.01 mg/kg	LM-MS/MS	Kang, J., 2003, B.5.2.1.3, DAR, Azoxystrobin – Methods of analysis
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Chaggar, S., 2004, B.5.2.1.4, DAR, Azoxystrobin – Method of analysis

~~For any special comments or remarkable points concerning the analytical methods for the determination of residues in plant matrices, please refer to Appendix 1.~~

zRMS comments:

According to the SANTE/2020/12830, Rev.2: A validation of the primary monitoring method in an independent laboratory (ILV) is required for the determination of residues in food of plant origin. The

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applicant did not provided data on ILV method for methods for the determination of residues in matrix of high acid and high oil content. However considering that this data was not provided during EU review, this data gap should be filled at the level of the active substance.

Table 5.3-35: Statement on extraction efficient

	Method for products of plant origin
Required, available from:	N/A Wilkinson, M.J., Hepburn, D.F., Joseph, R.S.I., 1994 EU agreed DAR, Vol. 3, B.5; UK, 05/2009
Not required, because:	Residues below LOQ

For the detailed evaluation of (additional) studies on extraction efficiency, it is referred to Appendix 1.

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 Azoxystrobin in animal matrices is given in the following tables. For the detailed evaluation of additional studies it is referred to Appendix 1.

Table 5.3-46: Validated methods for food and feed of animal origin (if appropriate)

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	0.001 mg/kg	GC-NPD	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A	N/A	
	Confirmatory (if required)	N/A	N/A	
Eggs	Primary	0.01 mg/kg	LC-MS/MS	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A	N/A	
	Confirmatory (if required)	N/A	N/A	
Muscle	Primary	0.01 mg/kg	LC-MS/MS	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A	N/A	
	Confirmatory (if required)	N/A	N/A	

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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
Fat	Primary	0.01 mg/kg	LC-MS/MS	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A g	N/A	
	Confirmatory (if required)	N/A	N/A	
Liver	Primary	0.01 mg/kg	LC-MS/MS	Ryan, J. and Sapiets, A. 1996, B.5.2.2, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A mg/kg	N/A	
	Confirmatory (if required)	N/A mg/kg	N/A	

~~For any special comments or remarkable points concerning the analytical methods for the determination of residues in animal matrices, please refer to Appendix 1.~~

Table 5.3-47: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	N/A
Not required, because:	~~Residues below LOQ~~ No additional residue trials or metabolism studies were conducted for this submission. Therefore no grown radiolabelled or non-radiolabelled material is available to investigate extraction efficiency.

~~For the detailed evaluation of (additional) studies on extraction efficiency please refer to Appendix 1.~~

zRMS comments:

According to the SANTE/2020/12830, Rev.2: A validation of the primary monitoring method in an independent laboratory (ILV) is required for the determination of residues in food of animal origin. The applicant did not provided data on ILV methods for methods for the determination of residues in food of animal origin. However considering that this data was not provided during EU review, this data gap should be filled at the level of the active substance.

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 azoxystrobin in soil is given in the following tables. ~~For the detailed evaluation of additional studies it is referred to Appendix 1.~~

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Table 5.3-58: Validated methods for soil (if appropriate)

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	LC-MS/MS	Johnson, R.I., et al., 2000, B.5.3.1.1, DAR, Azoxystrobin – Methods of analysis
Confirmatory	0.02 mg/kg	HPLC-UV	Johnson, R.I., et al., 2000, B.5.3.1.1, DAR, Azoxystrobin – Methods of analysis

For any special comments or remarkable points concerning the analytical methods for soil please refer to Appendix 1.

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 Azoxystrobin in surface and drinking water is given in the following tables. For the detailed valuation of additional studies it is referred to Appendix 1.

Table 5.3-69: Validated methods for water (if appropriate)

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	GC-MSD	Robinson, N.J., 2000, B.5.3.2.1, DAR, Azoxystrobin – Methods of analysis
	ILV	N/A	N/A	
	Confirmatory	0.1 µg/L	GC-MSD	Robinson, N.J., 2000, B.5.3.2.1, DAR, Azoxystrobin – Methods of analysis
Surface water	Primary	0.1 µg/L	GC-MSD	Robinson, N.J., 2000, B.5.3.2.1, DAR, Azoxystrobin – Methods of analysis
	Confirmatory	0.1 µg/L	GC-MSD	Robinson, N.J., 2000, B.5.3.2.1, DAR, Azoxystrobin – Methods of analysis

For any special comments or remarkable points concerning the analytical methods for water please refer to Appendix 1.

zRMS comments:

According to the SANTE/2020/12830, Rev.2: A validation of the primary monitoring method in an independent laboratory (ILV) is required for the determination of residues in drinking water. The applicant

did not provided data on ILV method for the method for the determination of residues in drinking water. However considering that this data was not provided during EU review, this data gap should be filled at the level of the active substance.

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 azoxystrobin in air is given in the following tables. For the detailed evaluation of additional studies please refer to Appendix 1.

Table 5.3-710: Validated methods for air (if appropriate)

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	3 µg/m ³	GS-MSD	Crawford, N., 2001, B.5.3.3.1, DAR, Azoxystrobin – Methods of analysis
Confirmatory	3 µg/m ³	GS-MSD	Crawford, N., 2001, B.5.3.3.1, DAR, Azoxystrobin – Methods of analysis

For any special comments or remarkable points concerning the analytical methods for air it is referred to Appendix 1.

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 azoxystrobin in body fluids and tissues is given in the following table. For the detailed evaluation of additional studies it is referred to Appendix 1.

Table 5.3-811: Methods for body fluids and tissues (if appropriate)

Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 µg/mL	LC-MS/MS	Gemrot F., 2011 IIIA 5.8, Registration Report Part B, Section 2: Analytical Methods, Syngenta, 16 May 2013
Confirmatory	0.01 µg/mL	LC-MS/MS	Gemrot F., 2011 IIIA 5.8, Registration Report Part B, Section 2: Analytical

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Component of residue definition: Azoxystrobin			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
			Methods, Syngenta, 16 May 2013

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 1.

5.3.2.8 Other studies/ information

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

Please refer to Appendix 1.

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

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

Table 5.3-94: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Difenoconazole	0.1 0.05 mg/kg	Reg. (EU) No 2019/552-2024/2612
Plant, high acid content	Difenoconazole	0.1 mg/kg	Reg. (EU) No 2019/552-2024/2612
Plant, high protein/high starch content (dry commodities)	Difenoconazole	0.05 mg/kg	Reg. (EU) No 2019/552-2024/2612
Plant, high oil content	Difenoconazole	0.05 mg/kg	Reg. (EU) No 2019/552-2024/2612
Muscle	Difenoconazole alcohol (CGA 205375) expressed as difenoconazole	0.05 mg/kg	Reg. (EU) No 2019/552-2024/2612
Milk	Difenoconazole alcohol (CGA 205375) expressed as difenoconazole	0.005 mg/kg	Reg. (EU) No 2019/552-2024/2612

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Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Eggs	Difenoconazole alcohol (CGA 205375) expressed as difenoconazole	0.05 mg/kg	Reg. (EU) No ~~2019/552~~ 2024/2612
Fat	Difenoconazole alcohol (CGA 205375) expressed as difenoconazole	0.05 mg/kg	Reg. (EU) No ~~2019/552~~ 2024/2612
Liver	Difenoconazole alcohol (CGA 205375) expressed as difenoconazole	0.1 mg/kg	Reg. (EU) No ~~2019/552~~ 2024/2612
Soil	Difenoconazole and CGA 205375	0.05 mg/kg	Common limit
Drinking water (Human toxicology)	Difenoconazole	0.1 µg/L	General limit for drinking water
Surface water (Ecotoxicology)	Difenoconazole	NOEC = 0.0056 mg a.s./L (regulatory concentration including a safety factor of 10 = 0.00056 mg a.s./L) Daphnia (<i>magna</i>) prolonged	
Air	Difenoconazole	~~0.99 ng/L~~ 48 µg/m ³	AOEL sys/AOEL inhal: 0.16 mg/kg bw/d
Tissue (meat or liver)	Difenoconazole	Analytical method is not required since difenoconazole is not classified as toxic or highly toxic. 0.01 mg/kg for tissue 0.01 mg/L for body fluids	EFSA Journal 2011;9(1):1967
Body fluids			SANTE/2020/12830, Rev.2

5.3.3.2 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 difenoconazole in plant matrices is given in the following tables. ~~For the detailed evaluation of additional studies it is referred to~~
~~Appendix 1.~~

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Table 5.3-15: 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: Difenoconazole				
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	LC-MS/MS	Steinhauer S. (2004a) , B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Schulz H. (2004), B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	Confirmatory (if required)	N/A	N/A	
High oil content	Primary	0.05 mg/kg	LC-MS/MS	Steinhauer S. (2004a) , B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Schulz H. (2004), B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	Confirmatory (if required)	N/A	N/A	
High protein/high starch content (dry)	Primary	0.05 mg/kg	LC-MS/MS	Steinhauer S. (2004a) , B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	ILV	0.01 mg/kg	LC-MS/MS	Schulz H. (2004), B.5.2.1, DAR, Difeconazole – Methods of analysis, EU agreed
	Confirmatory (if required)	N/A	N/A	

For any special comments or remarkable points concerning the analytical methods for the determination of residues in plant matrices, please refer to Appendix 1.

Table 5.3-10: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	N/A
Not required, because:	Residues below LOQ Extraction efficiency was not evaluated during the EU Review of Difenoconazole (EFSA Journal 2011;9(1):1967). This information will be submitted in a future in the renewal of the active substance.

zRMS comments:

According to the EFSA Journal 2017;15(7):4893: “Analytical methods for the determination of

difenoconazole residues in plant commodities were assessed during the peer review under Directive 91/414/EEC (Sweden 2006; EFSA, 2011a). The multiresidues DFG S19 method using liquid chromatography with tandem mass spectrometry (LC–MS/MS) was sufficiently validated at the LOQ of 0.02 mg/kg in high water content commodities (apple, lettuce) and at the LOQ of 0.05 mg/kg for dry (wheat grain) and high oil content commodities (rape seed).

The multiresidue Quick, Easy, Cheap, Effective, Rugged, and Safe (method) (QuEChERS) LC–MS/MS method described in the European Standard EN 15662:2008 is also applicable. The method analyses difenoconazole residues in high water content, acidic and dry commodities with an LOQ of 0.01 mg/kg (CEN, 2008). No validation data are available for spices, which do not belong to any of the group above. Considering that spices are minor crops the deviation is acceptable, the analytical methods validated for the four major crop groups are expected to apply to spices as well.”

5.3.3.3 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 difenoconazole in animal matrices is given in the following tables. For the detailed evaluation of additional studies it is referred to Appendix 1.

Table 5.3-16: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.005 mg/kg	LC-MS/MS	Crook S.J. (2004) , B.5.2.2., DAR, Difenoconazole – Methods of analysis Ryan J. (2004b).B.5.2.2., DAR, Difenoconazole – Methods of analysis
	ILV	0.005 mg/kg	LC-MS/MS	Benaseraf L. (2004), B.5.2.2., DAR, Difenoconazole – Methods of analysis
	Confirmatory (if required)	N/A	N/A	
Eggs	Primary	0.01 mg/kg	LC-MS/MS	Crook S.J. (2004) , B.5.2.2., DAR, Difenoconazole – Methods of analysis Ryan J. (2004b).B.5.2.2., DAR, Difenoconazole – Methods of analysis
	ILV	0.01 mg/kg	LC-MS/MS	Benaseraf L. (2004), B.5.2.2., DAR, Difenoconazole – Methods of analysis
	Confirmatory (if required)	N/A	N/A	
Muscle	Primary	0.01 mg/kg	LC-MS/MS	Crook S.J. (2004) , B.5.2.2., DAR, Difenoconazole – Methods

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Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
				of analysis Ryan J. (2004b).B.5.2.2., DAR, Difenoconazole – Methods of analysis
	ILV	0.01 mg/kg	LC-MS/MS	Benaseraf L. (2004), B.5.2.2., DAR, Difenoconazole – Methods of analysis
	Confirmatory (if required)	N/A	N/A	

Component of residue definition: Triazole Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid)				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Animal products, food of animal origin (residues)	Primary (honey)	0.01 mg/kg	LC-DMS- MS/MS	Mahlow S., Okeoghene Ererevwe K., 2023, S21-05867 Not presented on EU level
	ILV (honey)	0.01 mg/kg	LC-MS/MS	Kreidler D., 2024, S23-104559 Not presented on EU level
	Confirmatory (if required)	Same as primary.		

zRMS comments:

According to the EFSA Journal 2017;15(7):4893: “The analytical methods for the determination of difenoconazole residues in commodities of animal origin were evaluated during the peer review under Directive 91/414/EEC (Sweden, 2006; EFSA, 2011a). Difenoconazole and CGA-205375 can be determined by LC–MS/MS with and LOQ of 0.01 mg/kg in tissues, fat and eggs, and with an LOQ of 0.005 mg/kg in milk.

The multiresidue QuEChERS method is also applicable (CEN, 2008). Difenoconazole and CGA-205375 can be determined by LC–MS/MS with an LOQ of 0.01 mg/kg in tissues, fat and eggs, and with an LOQ of 0.005 mg/kg in milk per each analyte. EFSA concludes that sufficiently validated analytical methods for enforcing the MRLs for difenoconazole in food of animal origin are available.”

For any special comments or remarkable points concerning the analytical methods for the determination of residues in animal matrices, please refer to Appendix 1.

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Table 5.3-11: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	N/A
Not required, because:	Residues below LQ Extraction efficiency was not evaluated during the EU Review of Difenconazole (EFSA Journal 2011;9(1):1967). This information will be submitted in a future in the renewal of the active substance.

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

An overview on the acceptable methods and possible data gaps for analysis of difenoconazole in soil is given in the following tables. For the detailed evaluation of additional studies it is referred to Appendix 1.

Table 5.3-1217: Validated methods for soil (if appropriate)

Component of residue definition: Difenconazole and CGA 205375			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg	LC-MS/MS	Tummon O.J. (2004a), B.5.3.1, DAR, Difeconazole – Methods of analysis
Confirmatory	N/A	N/A	N/A

For any special comments or remarkable points concerning the analytical methods for soil please refer to Appendix 1.

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

An overview on the acceptable methods and possible data gaps for analysis of difenoconazole in surface and drinking water is given in the following tables. For the detailed valuation of additional studies it is referred to Appendix 1.

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Table 5.3-1318: Validated methods for water (if appropriate)

Component of residue definition: Difenoconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water	Primary	0.05 µg/L	GC-ECD	Tribolet R. (1999a), B.5.3.2., DAR, Method of analysis – Difenoconazole, Tribolet R. (1999b) B.5.3.2., DAR, Difeconazole – Methods of analysis
	ILV	N/A	N/A	
	Confirmatory	N/A	N/A	
Surface	Primary	0.1 µg/L	GC-ECD	Tribolet R. (1999a), B.5.3.2., DAR, Method of analysis – Difenoconazole, Tribolet R. (1999b) B.5.3.2., DAR, Difeconazole – Methods of analysis
	Confirmatory	0.1 µg/L	HPLC-UV	Tribolet R. (1999a), B.5.3.2., DAR, Method of analysis – Difenoconazole, Tribolet R. (1999b) B.5.3.2., DAR, Difeconazole – Methods of analysis

~~For any special comments or remarkable points concerning the analytical methods for water please refer to Appendix 1.~~

zRMS comments:

According to the SANTE/2020/12830, Rev.2: A validation of the primary monitoring method in an independent laboratory (ILV) is required for the determination of residues in drinking water. The applicant did not provided data on ILV method for the method for the determination of residues in drinking water. However considering that this data was not provided during EU review, this data gap should be filled at the level of the active substance.

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

An overview on the acceptable methods and possible data gaps for analysis of difenoconazole in air is given in the following tables. ~~For the detailed evaluation of additional studies please refer to Appendix 1.~~

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Table 5.3-1419: Validated methods for air (if appropriate)

Component of residue definition: Difenoconazole			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.99 ng/L	LC-MS/MS	Tummon O.J (2004b), B.5.3.4., DAR, Difeconazole – Methods of analysis
Confirmatory	N/A	N/A	

For any special comments or remarkable points concerning the analytical methods for air it is referred to Appendix 1.

5.3.3.7 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 difenoconazole in body fluids and tissues is given in the following table. For the detailed evaluation of additional studies it is referred to Appendix 1.

Table 5.3-1520: Methods for body fluids and tissues (if appropriate)

Component of residue definition: Difenoconazole			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	Analytical method is not required since difenoconazole is not classified as toxic or highly toxic.		
Confirmatory	N/A	N/A	

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 1.

zRMS comments:

According to the SANTE/2020/12830, Rev.2: 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. The applicant did not provided analytical methods for monitoring residues in body fluids and tissues. However considering that this data was not provided during EU review, this data gap should be filled at the level of the active substance.

5.3.3.8 Other studies/ information

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Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1	I.Knapik	2023	Validation of analytical method for CHR/F/SCORPIO 325 SC for determination of azoxystrobin, difenoconazole and impurities Z-azoxystrobin and toluene ICB Pharma 10 Lema Street 43-600, Jaworzno POLAND Study code: ICB/52/2023 No published GLP	N	Chemiro1
KCP 5.2/01 KCP 5.2/03	S.Niewelt- Stasiak	2023	Validation of an analytical method for the determination of residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves) SGS POLSKA Sp. z o.o. ul. Jana Kazimierza 3 01-248 Warszawa LABORATORIUM SGS POLSKA ul. Cieszyńska 52 A 43-200 Pszczyna Poland Validation Study No: VAL/22/2023 Not published GLP	N	Chemiro1
KCP 5.2/02 KCP 5.2/04	S.Mahlow C.Ezeobi-Chris	2023	Validation of Multi-Residue Method QuEChERS for Determination of Difenoconazole and Azoxystrobin in Honey Eurofins Agroscience Services Chem GmbH Am Neuländer Gewerbepark 2 21079 Hamburg Germany Study Code: S22-08436 (Int. Code GAB-2301V) Not published GLP	N	Chemiro1

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Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2/05	S. Niewelt-Stasiak	2022	Validation of an analytical method for the determination of residues of Triazole Derivative Metabolites (TAA, TA, 1,2,4-T, TLA) in Oilseed Rape (OSR) - seed, straw, plant SGS Polska Sp.z o.o. ul. Cieszyńska 52A 43-200 Pszczyna Poland Validation Study No: VAL/10/2021 GLP yes Not published	N	Chemiro1
KCP 5.2/06	S. Niewelt-Stasiak	2023	Validation of an analytical method for the determination of residues of Triazole Derivative Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid) in sugar beet (roots, leaves) Validation Study No: VAL/21/2023 GLP yes Not published	N	Chemiro1
KCP 5.2/07	S. Mahlow, K.O. Ererevwe	2023	Storage Stability of Triazole Metabolites in Honey under Deep Frozen Conditions Eurofins Agrosience Services Chem GmbH Am Neuländer Gewerbepark 2 21079 Hamburg Germany Study no: S21-05867 GLP Yes	N	Chemiro1
KCP 5.2/08	D. Kreidler	2024	Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Prothioconazole, Prothioconazole-desthio and Azoxystrobin in Honey and Pollen and QuPpe-PO-Method for Determination of 1,2,4 Triazole (T), Triazole Alanine (TA), Triazole Acetic Acid (TAA) and Triazole Lactic Acid (TLA) in Honey Eurofins Agrosience Services EAG Laboratories GmbH Eiselaer Weg 4, Geb./Bldg. 5 89081 Ulm Germany	N	Chemiro1

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Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Study Code: S23-104559 GLP yes		

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2	Robinson, N.J. et al	1999	Residue Analytical Method for the Determination of Azoxystrobin and R230310 in Crops Syngenta File No. ICI5504/1022 GLP: Yes Unpublished	N	Syngenta
KCP 5.1.2	Lister, N.	1999	Azoxystrobin: Validation of RAM 305/01 for the determination of Azoxystrobin and R230310 in Crops. Report No. RJ2770B Syngenta File No. ICI5504/0280 GLP Unpublished	N	Syngenta
KCP 5.2	Ryan, J. and Sapiets, A.	1996	ICIA5504 and R230310: Validation of a method for the determination of residues in animal tissue, eggs and milk. RAM 255/03 Report number: RJ1809B Syngenta File No. ICIA5504 and R230310		Syngenta

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Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Unpublished		
KCP 5.2	Johnson, R.I., et al	2000	Residue Analytical Method for the Analysis of Azoxystrobin, R230310, R2334886, R401553 and R402173 (in soil). RAM 269/03 Syngenta File No. ICI5504/0751 GLP: No Unpublished	N	Syngenta
KCP 5.2.2	Hall, M.G.	1999	Azoxystrobin and R234886: Determination in Human and Animal Plasma by LC-UV and LC-MS. 5.8, Registration Report Part B, Section 2: Analytical Methods, Syngenta, 16 May 2013		
KCP 5.2	Crawford, N.	2001	Azoxystrobin: Validation of an Analytical Method for the Determination of Residues in Air. RAM 376/01 Report number: TMJ4658B Syngenta File No. ICI5504/0011 No GLP Unpublished	N	Syngenta
KHIA 5.8/01	xxxxxxx	2011	Azoxystrobin Validation of analytical method RAM 399/01 for the determination of azoxystrobin, R230310 and R234886 in human whole blood. Syngenta xxxxxxx S10-03815 GLP, not published. Syngenta File No ICI5504_11467	Y	Syngenta
KCP 5.1	Chaggar, S.	2004	Residue Analytical Method for the Determination of Residues of Azoxystrobin (ICI5504)	N	Syngenta

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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
			and R230310 in Crop Samples. Report No. RAM 305/03 Syngenta File No. ICI5504/2686 GLP Yes Unpublished		
KCP 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 Root/Tuber Crops GLP: Yes Unpublished	N	Syngenta
KCP 5.2.2	xxxxxxx	2004	Residue Method for the Determination of Residues of Difenoconazole (CGA 169374) and CGA 205375 in Animal Products.Final Determination by LC-MS/MS xxxxxxx Report No REM 147.07 Not GLP Not published Syngenta File No CGA206375/0021	Y	Syngenta
KCP 5.2.2.	xxxxxxx	2004a	Difenoconazole (CGA 169374): Summary of Validation Data for Analytical Method RES10/93 on Various Crops xxxxxxx Report No TMJ4940B Not GLP Not Published Syngenta File No CGA169374/2421	Y	Syngenta
KCP 5.2	xxxxxxx	2004a	Difenoconazole. Validation of an Analytical Method for the Determination of Residues of Difenoconazole and CGA205375 in Soil xxxxxxx Report No RJ3459b	Y	Syngenta

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Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Not Published Syngenta File No CGA169374/2501		
KCP 5.3.3.5	Tribolet, R	1999a	Determination of parent compound by gas chromatography, Water Novartis Crop Protection, AG, Basel, Switzerland Nowartis Crop Protection, AG, Basel, Switzerland Raport No REM 147.05 Not GLP NoT Published Synegnta File No CGA169374/1783	N	Syngenta
KCP 5.3.3.5	Tribolet, R	1999b	Validation of Method REM 147.05 by Analysis of Fortfied Water Specimens for Difenoconazole (CGA 169374) and Evaluation of Recoveries Novartis Crop Protection AG, Basel Switzerland, Report No 226/98 GLP Not Published Syngenta File No CGA169374/1782	N	Syngenta
KCP 5.3.3.2	xxxxxxx	2004a	Difenoconazole: Validation of the DFG Method S 19 (Extended Revision) for the Determination of residues of Difenoconazole in Apple, Oil Seed Rape, Wheat (Grain) and Lettuce xxxxxxx Report No SYN-0301V A. G03-0012 GLP Not Published Syngenta File No CGA169374/2428	Y	Syngenta
KCP 5.3.3.3	xxxxxxx	2004	Independent Laboratory Validation of Residue Method REM 147.07 for the Determination of Difenoconazole and CGA205375 in Animal Products	Y	Syngenta

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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
			xxxxxxx Report No SYN/DIF/04031 GLP Not Published Syngenta File No CGA169374/2535		
KCP 5.3.3.1	xxxxxxx	2004	Independent Laboratory Validation of DFG Methos S19 (Extended Revision) for the Determination of Residues of difenoconazole in/on plant matrices xxxxxxx Report No IF-04/00160619 GLP Not Published Syngenta File No CGA169374/2507	Y	Syngenta

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for azoxystrobin and difenoconazole

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

Comments of zRMS	The objective of the study was to validate an analytical method for the determination of residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves). Specimen extraction and determination of residues was performed using QuEChERS technique. The specimens were prepared, extracted and analyzed following analytical procedures: - ANALYTICAL PROCEDURE DPL-65 Determination of difenoconazole and azoxystrobin in plant material using QuEChERS method and LC-MS/MS tandem mass spectrometry, based on: EN 15662:2018 Foods of plant origin. Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE. Modular QuEChERS-method. The limit of detection (LOD) in oilseed rape (grain, plant) was 0.002 mg/kg, in oilseed rape (straw) was 0.0025 mg/kg, in sugar beet (roots, leaves) was 0.001 mg/kg. The limit of quantification (LOQ) of the analytical method was 0.010 mg/kg for difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves). The total analytical procedure for each matrices was performed and completed within 1 day. For analyte, two mass transitions were evaluated and used for quantification. Representative chromatograms are shown in the report. Optionally, a third mass transition was monitored for confirmation of peak identity, but was not used for quantification. Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). An average recovery in the range of 70 – 120% and RSD ≤ 20 % was achieved. The analytical method for determining the residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves) meets the criteria of SANTE/2020/12830 Rev.2 in terms of precision, accuracy and uncertainty. The study is accepted.
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Reference:

KCP 5.2/01

Report:

Validation of an analytical method for the determination of residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves), PROJECT CODE: VAL/22/2023

Guideline(s):

Regulation (EC) No. 1107/2009

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SANTE/2020/12830 Rev.2

Deviations:	NO
GLP:	YES
Acceptability:	YES

Materials and methods

The objective of the study was to validate an analytical method for the determination of residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves).

The limit of detection (LOD) in oilseed rape (grain, plant) that was expressed as the lowest calibration standard was 0.002 mg/kg, what corresponds to 0.001 µg/mL (because weigh of sample was equal 5 g), in oilseed rape (straw) LOD that was expressed as the lowest calibration standard was 0.0025 mg/kg, what corresponds to 0.0005 µg/mL µg/mL (because weigh of sample was equal 2 g), in sugar beet (roots, leaves) LOD that was expressed as the lowest calibration standard was 0.001 mg/kg, what corresponds to 0.001 µg/mL µg/mL (because weigh of sample was equal 10 g). The limit of quantification (LOQ) of the analytical method was 0.010 mg/kg for difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves).

Specimen preparation

10 g (roots, leaves) / 5 g (seed, plant) / 2 g (straw) of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of deionized water (in case of seed, plant, straw) and 10 mL of acetonitrile (with 1% of formic acid) was added together with 100 µL (roots, leaves) / 50 µL (grain, plant) / 20 µL (straw) of internal standard solution (1.4 and 1.5), and the mixture was shaken vigorously by hand for one minute. After addition of buffering salts (4 g anhydrous magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dehydrate, 0.5 g disodium hydrogencitrate sesquihydrate), the mixture was shaken again intensively for 1 min, then centrifuged at 4700 rpm for 5 min for phase separation and finally subjected to a freezing process at ≤ -18°C for 1.5 h. After that, 5 mL of the clean extract (organic phase) was transferred to a 10 mL vial. Following clean-up the extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis. Quantification was performed using an internal standard, which was added to the extract after the initial addition of acetonitrile (with 1% of formic acid).

Equipment and reagents

- Electronic balance Class I; WP-011/W/S/LF
- Electronic balance Class II; WP-012/W/S/LF, WP-013/W/S/LF
- Freezers - storage of:
 - analytical standards; W-082/S/LF
 - analytical samples before the analytical part; W-093/LF
 - analytical samples extracts–freezing during extraction/preparation of samples; W084/S/LF
 - analytical samples extracts until the end of the instrumental analysis; W-084/S/LF
- Centrifuge (> 3000 rpm); W-076/LF
- Polypropylene centrifuge tubes, with screw caps, 50 mL
- Plastic cups (stackable), 100 mL, for the storage of archived samples
- Volumetric flasks, 5 and 10 mL (class A)
- Automatic pipettes: 10 – 200 µL (WP-137/S/LF); 0.05-1.00 mL (WP-138/S/LF); 0.5-5.00 mL (WP-102/S/LF); 1.00-10.0 mL (WP-103/S/LF)
- Vials with screw caps, amber glass, 10 mL

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- Injection vials, 1.5 mL, amber glass, screw caps with septa butyl/PTFE
- Liquid Chromatography Instrument LC-MSMS Shimadzu 8050; WP-135/K/S/ko/LF
- Syringe filters RC 0.22 µm, 25 mm

REAGENTS

- Deionized Water, Lot: 23H304043
- Acetonitrile, CH₃CN, for HPLC, Lot: 23G134012
- Acetonitrile, CH₃CN, hypergrade for LC-MS, Lot: J1273629310
- Formic acid, HCOOH, 98 – 100%, for analysis, Lot: 22D114007
- Buffer salt mixture, Lot: 4423, containing:
 - 4g ± 0.2g anhydrous magnesium sulfate
 - 1 g ± 0.05g sodium chloride
 - 1 g ± 0.05g trisodium citrate dehydrate
 - 0.50g ± 0.03g disodium hydrogencitrate sesquihydrate

Chromatographic parameters

Instrument settings:

Liquid Chromatograph LCMS-8050 Shimadzu (WP-135/K/S/ko/LF), consists of:

Degazer DGU-403

Two pumps LC-40D XR

Autosampler SIL-40C XR

Column oven CTO-40S

Compressor, generator PEAK Genius 1051

HPLC Column – Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, Lot: B20115

Pumps:

Mode – Binary gradient

Total Flow – 0.3 mL/min

Mobile Phase A – 0.1% formic acid in deionized water

Mobile Phase B – 0.1% formic acid in acetonitrile

A Conc – 80 %

B Conc – 20%

Time	Module	Command	Value %
0.01	Pumps	Pump B Conc.	20
10.00	Pumps	Pump B Conc.	90
14.00	Pumps	Pump B Conc.	95
14.01	Pumps	Pump B Conc.	20
16.00	Pumps	Pump B Conc.	20
16.00	Controller	Stop	

Cooler Temp.: 4°C

Oven Temp.: 40°C

Interface: ESI

Interface heater: on

Interface Temp.: 300 °C

DL Temperature: 250 °C

Nebulizing Gas Flow: 3.00 L/min

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Heating Gas: On
Heating Gas Flow: 10.0 L/min
Heat Block: 400 °C
Drying Gas: On
Drying Gas Flow: 10.0 L/min

Difenoconazole

Acquisition Mode: MRM
Polarity: Positive
Start time: 5.00 min
End Time: 14.00 min
Retention Time: 10.62 min

Difenoconazole D6 – internal standard

Acquisition Mode: MRM
Polarity: Positive
Start time: 5.00 min
End Time: 14.00 min
Retention Time: 10.60 min

Azoxystrobin

Acquisition Mode: MRM
Polarity: Positive
Start time: 5.00 min
End Time: 14.00 min
Retention Time: 9.10 min

Azoxystrobin D4 – internal standard

Acquisition Mode: MRM
Polarity: Positive
Start time: 5.00 min
End Time: 14.00 min
Retention Time: 9.08 min

Results and discussions

Table A-16: Recovery results from method validation of Azoxystrobin and Difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Oilseed rape seed	Difenoconazole 406.00→250.90	0.010	97.0	0.76	-
	Difenoconazole 406.00→250.90	0.10	96.4	1.37	-
	Difenoconazole 406.00→187.90	0.010	96.6	1.32	-
	Difenoconazole 406.00→187.90	0.10	96.4	1.37	-
	Azoxystrobin	0.010	94.0	0.92	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	404.00→372.05				
	Azoxystrobin 404.00→372.05	0.10	94.5	2.09	-
	Azoxystrobin 404.00→344.05	0.010	95.9	2.60	-
	Azoxystrobin 404.00→344.05	0.10	95.5	2.13	-
Oilseed rape straw	Difenoconazole 406.00→250.90	0.010	88.2	13.71	-
	Difenoconazole 406.00→250.90	0.10	98.0	1.56	-
	Difenoconazole 406.00→187.90	0.010	89.7	15.49	-
	Difenoconazole 406.00→187.90	0.10	98.9	1.78	-
	Azoxystrobin 404.00→372.05	0.010	92.3	16.60	-
	Azoxystrobin 404.00→372.05	0.10	96.6	2.30	-
	Azoxystrobin 404.00→344.05	0.010	93.0	17.79	-
	Azoxystrobin 404.00→344.05	0.10	98.2	2.50	-
Oilseed rape plant	Difenoconazole 406.00→250.90	0.010	102.7	1.65	-
	Difenoconazole 406.00→250.90	0.10	101.4	2.08	-
	Difenoconazole 406.00→187.90	0.010	101.8	1.40	-
	Difenoconazole 406.00→187.90	0.10	99.8	1.76	-
	Azoxystrobin 404.00→372.05	0.010	99.5	0.62	-
	Azoxystrobin 404.00→372.05	0.10	97.2	2.22	-
	Azoxystrobin 404.00→344.05	0.010	99.5	2.21	-
	Azoxystrobin 404.00→344.05	0.10	96.7	1.53	-
Sugar beet	Difenoconazole 406.00→250.90	0.010	99.7	1.12	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
leaves	Difenoconazole 406.00→250.90	0.10	99.3	0.92	-
	Difenoconazole 406.00→187.90	0.010	99.3	1.22	-
	Difenoconazole 406.00→187.90	0.10	99.6	1.08	-
	Azoxystrobin 404.00→372.05	0.010	93.9	0.43	-
	Azoxystrobin 404.00→372.05	0.10	98.2	1.79	-
	Azoxystrobin 404.00→344.05	0.010	95.9	1.24	-
	Azoxystrobin 404.00→344.05	0.10	98.6	1.46	-
Sugar beet roots	Difenoconazole 406.00→250.90	0.010	99.8	1.09	-
	Difenoconazole 406.00→250.90	0.10	102.4	2.05	-
	Difenoconazole 406.00→187.90	0.010	102.5	1.11	-
	Difenoconazole 406.00→187.90	0.10	103.5	2.03	-
	Azoxystrobin 404.00→372.05	0.010	97.0	1.75	-
	Azoxystrobin 404.00→372.05	0.10	95.4	2.11	-
	Azoxystrobin 404.00→344.05	0.010	96.3	2.14	-
	Azoxystrobin 404.00→344.05	0.10	94.9	2.41	-

Table A-1: Characteristics for the analytical method used for validation of Azoxystrobin and Difenoconazole residues in oilseed rape and sugar beets

	Residues
Author, year	S. Niewelt-Stasiak, 2023
Principle of method	LC MS/MS
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	The linearity of the detector response was demonstrated by single determination of calibration standards at six concentration levels ranging from 0.5 (oilseed rape straw) or 1.0 (oilseed rape – seed, plant, sugar beet – roots, leaves) to 500 ppb for difenoconazole and azoxystrobin in each matrix. The coefficient of determination (R ²) were determined. Linear

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	Residues
	regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than or equal to 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration
Recovery Accuracy Precision and repeatability	The LOQ is the lowest validated fortification level for which an average recovery in the range of 70 – 120% (60 – 120 % in case of level $\leq$ 0.01 mg/kg) and RSD $\leq$ 20 % ($\leq$ 30 % in case of level $\leq$ 0.01 mg/kg) is achieved.
Assessment of matrix effects is presented	For difenoconazole in oilseed rape (seed), difenoconazole in sugar beet (root) and azoxystrobin in oilseed rape (seed, straw) matrix effect calculated using equation is $< \pm 20\%$. In the rest of studied cases matrix effect exceed $\pm 20\%$. However to compensate matrix effects, the matrix-matched calibrations was used in case of each matrix. Based on the background verification of the chromatograms and possible interference at the retention time of the peak of the determined substance, it was found that all matrices meet the
LOQ	The limit of quantification (LOQ) of the analytical method was 0.010 mg/kg for difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves).
Comment	The validation parameters are within the acceptance range and fulfil EU requirements.

Conclusion

The analytical method for determining the residues of difenoconazole and azoxystrobin in oilseed rape (seed, plant, straw) and sugar beet (roots, leaves) meets the criteria of SANTE/2020/12830 Rev.2, 14 February 2023 documents in terms of precision, accuracy and uncertainty. The method was validated over the concentration range of 0.010-0.10 mg/kg for each matrix with a limit of quantification of 0.010 mg/kg for each of them. Limit of detection was established at 0.002 mg/kg for oilseed rape (seed, plant), at 0.0025 mg/kg for oilseed rape (straw) and at 0.001 mg/kg for sugar beet (roots, leaves).

Comments of zRMS	LC-MS/MS determination was conducted with evaluation of two (2) mass transitions for difenoconazole (m/z 406 $\rightarrow$ 251 and m/z 406 $\rightarrow$ 188) and for azoxystrobin (m/z 404 $\rightarrow$ 372 and m/z 404 $\rightarrow$ 344). Mass transition m/z 406 $\rightarrow$ 251 is proposed to be used for quantification for difenoconazole, but both mass transitions are applicable interchangeably for quantification and confirmation. Mass transition m/z 404 $\rightarrow$ 372 is proposed to be used for quantification for azoxystrobin, but both mass transitions are applicable interchangeably for quantification and confirmation. Matrix suppression or enhancement was $< 20\%$ for both analytes and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.
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	The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/kg was confirmed for difenoconazole and azoxystrobin in honey. The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for the matrix, which is 30 % of the LOQ. Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. For all analytes the mean recoveries were within 70 % – 110 %. The relative standard deviations overall testing intervals were ≤ 20 % for all analytes. The analytical method for determining the residues of difenoconazole and azoxystrobin in honey meets the criteria of SANTE/2020/12830 Rev.2 in terms of precision, accuracy and uncertainty. The study is accepted.
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Reference:	KCP 5.2/02
Report	Validation of Multi-Residue Method QuEChERS for Determination of Difenoconazole and Azoxystrobin in Honey. Study code: S22-08436
Guideline(s):	Regulation (EC) No. 1107/2009 SANTE/2020/12830, rev.2 OECD 506, 2007 7032/VI/95, rev. 5 1607/VI/97, rev. 2 ENV/JM/MONO(2007)17
Deviations:	NO
GLP:	YES
Acceptability:	YES

Materials and methods

To validate the multi-residue method QuEChERS for the determination of difenoconazole and azoxystrobin in honey in accordance to guidance document SANTE/2020/12830, rev.2.

Reagents and Materials:

- Formic acid
(Riedel de Haen, Art. No. 33015)
- Water
(VWR, HiPerSolv CHROMANORM for HPLC, Art. No. 23595.328)
- Acetonitrile 99.9 %
(VWR, Art. No. 20060.320)
- Disodium hydrogen citrate 1,5 Hydrate
(Merck KGaA, Art. No. 1.12264.0500)
- Magnesium sulphate 98.0%
(VWR, Art. No. 291186R)
- PSA polymerically bonded, ethylenediamine-N-propyl phase with primary and secondary amines
(Bondesil-PSA 40UM, Varian, Art. No. 12213025)
- Sodium chloride 99.9%
(VWR, Art. No. 27810.295)
- Trisodium citrate dihydrate

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(VWR Chemicals, Art. No. 27833.294)

- Formic acid 99.9 %

(HiPerSolv CHROMANORM® for LC-MS, VWR, Art. No. 84865.180)

Identification of Instrumentation and Apparatus

- Adjustable pipettes

(Reference 10-100 µL and Reference, 100-1000 µL, Eppendorf and HandyStep with 12.5 mL tip, Brand)

- Common laboratory glassware

- Centrifuge tubes with caps, PP, 50 mL (Sarstedt AG & Co., Art. No. 62547254)

- HPLC Autosampler vials, 1.8 mL

- Laboratory centrifuge for 50 mL centrifuge tubes

(Rotina Hettich 420R, Andreas Hettich GmbH & Co.KG)

- Platform shaker (SM 30 C, Edmund Bühler GmbH, Art. No. 6105 000)

- Test tubes, with ground stoppers and graduation marks at 2.5, 5.0 and 10 mL

- Volumetric pipettes, e.g., 1, 2 and 5 mL

- Vortex mixer (Reax TOP, HEIDOLPH Instruments GmbH & Co. KG, Art. No. 541-10000-00-1)

- Ultrasonic bath (Bandelin Sonorex RK 510 H, BANDELIN electronic GmbH & Co. KG, Art. No. 321)

- Safe lock tubes 2 mL (Eppendorf AG, Art. No. 0030120094)

- Microcentrifuge for 2 mL safe lock tubes 6000 U/min (Heraeus Pico 17, Thermo Fisher Scientific)

- Luna C18(2) 100A (50 mm x 3 mm, 3 µm)

(Phenomenex, Art. No. 00B-4251-Y0)

Method Summary:

Homogenisation: Stirring with a spatula thoroughly.

Extraction: Acetonitrile and addition of water

Ratio: 10 mL of extraction solvent per g of matrix

Liquid/liquid partition: Addition of magnesium sulphate, sodium chloride and sodium citrate followed by subsequent centrifugation

Clean-up: Dispersive SPE with primary/secondary amine (PSA)

Sample concentration in final extract: 0.05 g sample per mL of extract

Quantification: LC-MS/MS

Summary of chromatographic and mass spectrometric conditions:

Difenoconazole and Azoxystrobin

Chromatographic conditions						
HPLC system	1200 Binary Rapid Resolution LC System, Agilent Technologies (HPLC, ≤ 600 bar)					
Pre-column	Not used					
Column	Luna C18 (2) (50 mm x 3 mm, 3 µm, (Phenomenex, Art. No. 00B-4251-Y0)					
Column oven temperature	40 °C					
Injection volume	1.0 µL					
Mobile phases	Eluent A: Acetonitrile with 0.1 % (v/v) formic acid Eluent B: Water with 0.1 % formic acid					
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]		
	0.0	30	70	750		
	3.5	95	5.0	750		
	5.0	95	5.0	750		
	5.1	30	70	750		
	7.0	30	70	750		
Divert valve	0.0 min to 1.5 min to waste; 1.5 min to 3.8 min to MS; 3.8 min to 7.0 min to waste					
Retention time(s)	Difenoconazole: approx. 3.1 min Azoxystrobin: approx. 2.6 min					
Mass spectrometric conditions						
MS system	API 5000 System, SCIEX (Triple quadrupole mass spectrometer)					
Ionisation type	Electrospray ionisation (ESI, Turbo Ion Spray)					
Polarity	Positive					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	5500 V	Ionspray turbo heater (TEM)		400 °C		
Curtain gas (CUR)	30 (arbitrary units)	Gas flow 1 (GS1)		40 (arbitrary units)		
Collision gas (CAD)	7 (arbitrary units)	Gas flow 2 (GS2)		60 (arbitrary units)		
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
Difenoconazole	406 → 251* (pos)	80	10	39	14	50
	406 → 188 (pos)	80	10	63	22	75
Azoxystrobin	404 → 372* (pos)	40	10	20	10	50
	404 → 344 (pos)	40	10	35	10	50

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

Procedure for Honey in Difenoconazole and Azoxystrobin:

Extraction and Liquid/Liquid Partition

- 1) An amount of 1 g ± 0.01 g of homogenised sample was weighed into a 50-mL centrifuge tube.
- 2) 5.0 mL of water was added. The centrifuge tube was capped and shaken.
- 3) For extraction, exactly 10 mL of acetonitrile were added (VEx). The centrifuge tube was capped and shaken on a platform shaker for 15 minutes.
- 4) Thereafter, 4.0 g of magnesium sulphate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate were added. The centrifuge tube was capped again and immediately shaken by hand for one (1) minute.
- 5) The sample tube was centrifuged for about 5 minutes at about 3200 x g.

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Clean-up

1) An amount of 37.5 mg of PSA and 225 mg of magnesium sulphate was weighed into a 2-mL safelock tube. An aliquot of 1.5 mL of the upper acetonitrile phase was transferred into the tube containing the mixture of sorbents. The tube was intensively shaken by hand for 30 seconds followed by centrifugation for 5 minutes at about 3500 x g.

Final Fill-up for Analysis

2) Before analysis, exactly 0.50 mL of the purified extract (V_{Ali}) was diluted to 1.0 mL with 0.1 % formic acid in water and mixed using a Vortex mixer (V_{End} = 1.0 mL). The final extract was transferred to an HPLC vial and kept at typically 1 °C – 10 °C in the dark until injection to LC-MS/MS

Table B 2: Recovery results from confirmatory method validation of analyte using the confirmatory analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Honey	Difenoconazole 406→251	0.010	106	3.7	-
	Difenoconazole 406→251	0.10	104	4.3	-
	Difenoconazole 406→188	0.010	103	5.0	-
	Difenoconazole 406→188	0.10	93	4.1	-
	Azoxystrobin 404→372	0.010	100	7.0	-
	Azoxystrobin 404→372	0.10	101	5.7	-
	Azoxystrobin 404→344	0.010	96	5.0	-
	Azoxystrobin 404→344	0.10	93	8.4	-

Table B-17: Methods suitable for the determination of the residues in plant protection product CHR/F/SCORPIO 325

	Residues
Author(s), year	S. Mahlow, C. Ezeobi-Chris
Principle of method	LC MS/MS
Linearity (linear between mg/L) (correlation)	The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.15 ng/mL to 15 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract.

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	Residues																																																																																																																					
coefficient, expressed as r)																																																																																																																						
Recovery	<table><tr><th colspan="8">Difenoconazole</th></tr><tr><th>Matrix</th><th>Fortification Level (mg/kg)</th><th>Recovery (%)</th><th>Mean Recovery (%)</th><th>Rel. Std. Dev. (%)</th><th>Replicates</th><th>Overall Mean Recovery (%)</th><th>Overall Rel. Std. Dev. (%)</th></tr><tr><th colspan="8">Mass Transition <i>m/z</i> 406→251 (Proposed for Quantification)</th></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>102, 102, 110, 110, 104</td><td>106</td><td>3.7</td><td>5</td><td rowspan="2">105</td><td rowspan="2">3.9</td></tr><tr><td>0.10</td><td>100, 110, 99, 106, 103</td><td>104</td><td>4.3</td><td>5</td></tr><tr><th colspan="8">Mass Transition <i>m/z</i> 406→188 (Proposed for Confirmation)</th></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>103, 94, 107, 102, 107</td><td>103</td><td>5.0</td><td>5</td><td rowspan="2">98</td><td rowspan="2">6.6</td></tr><tr><td>0.10</td><td>100, 93, 90, 91, 93</td><td>93</td><td>4.1</td><td>5</td></tr><tr><th colspan="8">Azoxystrobin</th></tr><tr><th colspan="8">Mass Transition <i>m/z</i> 404→372 (Proposed for Quantification)</th></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>101, 100, 91, 99, 110</td><td>100</td><td>7.0</td><td>5</td><td rowspan="2">101</td><td rowspan="2">6.0</td></tr><tr><td>0.10</td><td>103, 93, 109, 102, 99</td><td>101</td><td>5.7</td><td>5</td></tr><tr><th colspan="8">Mass Transition <i>m/z</i> 404→344 (Proposed for Confirmation)</th></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>94, 103, 98, 92, 91</td><td>96</td><td>5.0</td><td>5</td><td rowspan="2">94</td><td rowspan="2">6.6</td></tr><tr><td>0.10</td><td>92, 103, 87, 99, 85</td><td>93</td><td>8.4</td><td>5</td></tr></table> No residue above 30 % of the LOQ level was detected in any control sample extract. Recoveries are corrected for the (mean) peak area(s) of the control sample extract(s). All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2. <table><tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr><tr><td>≤ 0.01</td><td>60 – 120</td><td>≤ 30</td></tr><tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr></table>	Difenoconazole								Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Mass Transition <i>m/z</i> 406→251 (Proposed for Quantification)								Honey	0.010	102, 102, 110, 110, 104	106	3.7	5	105	3.9	0.10	100, 110, 99, 106, 103	104	4.3	5	Mass Transition <i>m/z</i> 406→188 (Proposed for Confirmation)								Honey	0.010	103, 94, 107, 102, 107	103	5.0	5	98	6.6	0.10	100, 93, 90, 91, 93	93	4.1	5	Azoxystrobin								Mass Transition <i>m/z</i> 404→372 (Proposed for Quantification)								Honey	0.010	101, 100, 91, 99, 110	100	7.0	5	101	6.0	0.10	103, 93, 109, 102, 99	101	5.7	5	Mass Transition <i>m/z</i> 404→344 (Proposed for Confirmation)								Honey	0.010	94, 103, 98, 92, 91	96	5.0	5	94	6.6	0.10	92, 103, 87, 99, 85	93	8.4	5	Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)	≤ 0.01	60 – 120	≤ 30	> 0.01 - ≤ 0.1	70 - 120	≤ 20
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	Residues																																																																																																												
Accuracy	<table><tr><th colspan="8">Difenoconazole</th></tr><tr><th>Matrix</th><th>Fortification Level (mg/kg)</th><th>Recovery (%)</th><th>Mean Recovery (%)</th><th>Rel. Std. Dev. (%)</th><th>Replicates</th><th>Overall Mean Recovery (%)</th><th>Overall Rel. Std. Dev. (%)</th></tr><tr><td colspan="8">Mass Transition <i>m/z</i> 406→251 (Proposed for Quantification)</td></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>102, 102, 110, 110, 104</td><td>106</td><td>3.7</td><td>5</td><td rowspan="2">105</td><td rowspan="2">3.9</td></tr><tr><td>0.10</td><td>100, 110, 99, 106, 103</td><td>104</td><td>4.3</td><td>5</td></tr><tr><td colspan="8">Mass Transition <i>m/z</i> 406→188 (Proposed for Confirmation)</td></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>103, 94, 107, 102, 107</td><td>103</td><td>5.0</td><td>5</td><td rowspan="2">98</td><td rowspan="2">6.6</td></tr><tr><td>0.10</td><td>100, 93, 90, 91, 93</td><td>93</td><td>4.1</td><td>5</td></tr><tr><td colspan="8">Azoxystrobin</td></tr><tr><td colspan="8">Mass Transition <i>m/z</i> 404→372 (Proposed for Quantification)</td></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>101, 100, 91, 99, 110</td><td>100</td><td>7.0</td><td>5</td><td rowspan="2">101</td><td rowspan="2">6.0</td></tr><tr><td>0.10</td><td>103, 93, 109, 102, 99</td><td>101</td><td>5.7</td><td>5</td></tr><tr><td colspan="8">Mass Transition <i>m/z</i> 404→344 (Proposed for Confirmation)</td></tr><tr><td rowspan="2">Honey</td><td>0.010</td><td>94, 103, 98, 92, 91</td><td>96</td><td>5.0</td><td>5</td><td rowspan="2">94</td><td rowspan="2">6.6</td></tr><tr><td>0.10</td><td>92, 103, 87, 99, 85</td><td>93</td><td>8.4</td><td>5</td></tr></table> No residue above 30 % of the LOQ level was detected in any control sample extract. Recoveries are corrected for the (mean) peak area(s) of the control sample extract(s).	Difenoconazole								Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	Mass Transition <i>m/z</i> 406→251 (Proposed for Quantification)								Honey	0.010	102, 102, 110, 110, 104	106	3.7	5	105	3.9	0.10	100, 110, 99, 106, 103	104	4.3	5	Mass Transition <i>m/z</i> 406→188 (Proposed for Confirmation)								Honey	0.010	103, 94, 107, 102, 107	103	5.0	5	98	6.6	0.10	100, 93, 90, 91, 93	93	4.1	5	Azoxystrobin								Mass Transition <i>m/z</i> 404→372 (Proposed for Quantification)								Honey	0.010	101, 100, 91, 99, 110	100	7.0	5	101	6.0	0.10	103, 93, 109, 102, 99	101	5.7	5	Mass Transition <i>m/z</i> 404→344 (Proposed for Confirmation)								Honey	0.010	94, 103, 98, 92, 91	96	5.0	5	94	6.6	0.10	92, 103, 87, 99, 85	93	8.4	5
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Azoxystrobin																																																																																																													
Mass Transition <i>m/z</i> 404→372 (Proposed for Quantification)																																																																																																													
Honey	0.010	101, 100, 91, 99, 110	100	7.0	5	101	6.0																																																																																																						
	0.10	103, 93, 109, 102, 99	101	5.7	5																																																																																																								
Mass Transition <i>m/z</i> 404→344 (Proposed for Confirmation)																																																																																																													
Honey	0.010	94, 103, 98, 92, 91	96	5.0	5	94	6.6																																																																																																						
	0.10	92, 103, 87, 99, 85	93	8.4	5																																																																																																								
Precision and repeatability	<table><tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr><tr><td>≤ 0.01</td><td>60 – 120</td><td>≤ 30</td></tr><tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr></table>	Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)	≤ 0.01	60 – 120	≤ 30	> 0.01 - ≤ 0.1	70 - 120	≤ 20																																																																																																			
Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)																																																																																																											
≤ 0.01	60 – 120	≤ 30																																																																																																											
> 0.01 - ≤ 0.1	70 - 120	≤ 20																																																																																																											
Specificity	Demonstrated by validation of two (2) mass transitions. Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.																																																																																																												
Recoveries	<table><tr><th rowspan="2">Analyte</th><th rowspan="2">Fortification level (mg/kg)</th><th colspan="3">Recovery (%)</th><th rowspan="2">Mean (%)</th><th rowspan="2">Rel. Std. Dev. (%)</th></tr><tr><th>Day 0 Testing*</th><th>1 month</th><th>3 months</th></tr><tr><td colspan="7">Honey</td></tr><tr><td>Difenoconazole (<i>m/z</i> 406→251)</td><td>0.10</td><td>94, 95</td><td>70, 70</td><td>90, 94</td><td>86</td><td>14</td></tr><tr><td>Azoxystrobin (<i>m/z</i> 404→372)</td><td>0.10</td><td>94, 83</td><td>75, 78</td><td>98, 90</td><td>86</td><td>11</td></tr></table> * Day 0 storage samples included For all analytes the mean recoveries were within 70 % – 110 %. The relative standard deviations over all testing intervals were ≤ 20 % for all analytes.	Analyte	Fortification level (mg/kg)	Recovery (%)			Mean (%)	Rel. Std. Dev. (%)	Day 0 Testing*	1 month	3 months	Honey							Difenoconazole (<i>m/z</i> 406→251)	0.10	94, 95	70, 70	90, 94	86	14	Azoxystrobin (<i>m/z</i> 404→372)	0.10	94, 83	75, 78	98, 90	86	11																																																																													
Analyte	Fortification level (mg/kg)			Recovery (%)					Mean (%)	Rel. Std. Dev. (%)																																																																																																			
		Day 0 Testing*	1 month	3 months																																																																																																									
Honey																																																																																																													
Difenoconazole (<i>m/z</i> 406→251)	0.10	94, 95	70, 70	90, 94	86	14																																																																																																							
Azoxystrobin (<i>m/z</i> 404→372)	0.10	94, 83	75, 78	98, 90	86	11																																																																																																							
Matrix Effects on Analyte Detection I	Insignificant (< 20 %) for both analytes																																																																																																												
LOQ	0.01 mg/kg (lowest validated fortification level)																																																																																																												
Comment	With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the samples of the study. For storage stability testing, the average amount of analyte recovered relative to the initial mean recovery at day 0 was ≥ 70 % at a tested storage interval of 104 days. The study is deemed sufficient for assessing the stability of difenoconazole and azoxystrobin in honey upon storage at ≤ -18 °C for 104 days																																																																																																												

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Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring

Comments of zRMS	The purpose of this study was to validate an analytical method for the determination of residues of triazole derivative metabolites (TDMs = 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid, Triazole-lactic acid) in oilseed rape (OSR) - seed, straw, plant. Specimen extraction and determination of residues of TDMs was performed using the QuPpe method. Quantification was performed by use of LC-MS/MS detection system. The limit of detection (LOD) and quantification (LOQ) of the analytical method were subsequently: LOD: 0.004 mg/kg (seed, plant), 0.005 mg/kg (straw) and LOQ: 0.010 mg/kg for TAA, TA, 1,2,4-T, TLA in seed, plant and straw. For each analyte, two mass transitions were evaluated and used for quantification. Representative chromatograms were shown in the report. A third mass transition was monitored for confirmation of peak identity, but was not used for quantification. Referring to the requirements of SANTE/2020/12830 if the matrix effect exceeds 20% LOQ, the "matrix-matched" calibration should be introduced. This effect was not observed during validation, but the method of preparing working calibration standards based on blank sample was used. Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. For all analytes the mean recoveries were within 70 % – 120 %. The relative standard deviations overall testing intervals were ≤ 20 % for all analytes. The analytical method for determining the residues of Triazole Derivative Metabolites (TDMs = 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid, Triazole-lactic acid) in oilseed rape (seed, straw and plant) meets the criteria of SANTE/2020/12830, Rev.1 in terms of precision, accuracy and uncertainty. The study is accepted.
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Reference: KCP 5.2/05

Report Validation of an analytical method for the determination of residues of Triazole Derivative Metabolites (TAA, TA, 1,2,4-T, TLA) in Oilseed Rape (OSR) - seed, straw, plant, Validation Study No: VAL/10/2021

Guideline(s): Regulation (EC) No 1107/2009
SANTE/2020/12830 Rev.1

Deviations: NO

GLP: YES

Acceptability: YES

Materials and methods

The method for the determination of residues of TAA, TA, 1,2,4-T, TLA was validated in oilseed rape (seed, straw, plant). The method was validated over the concentration range of 0.010-0.10 mg/kg (µg/g) with an estimated limit of detection at 0.004 mg/kg (seed, plant) and 0.005 mg/kg (straw).

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Reagents

- Deionized Water
- Acetonitrile, CH₃CN, for GC applications (pesticide residues analysis), Lot: 20L152008
- Methanol, CH₃OH, HPLC grade, Lot: 211018A001
- Formic Acid, HCOOH, > 98%, p.a. Lot: 21A204018
- Acetic Acid, CH₃COOH, > 98%, p.a., Lot: 19E024015

Equipment

- Electronic balance Class I; WP-011/W/S/LF
- Electronic balance Class II; WP-012/W/S/LF, WP-013/W/S/LF
- Freezers - storage of:
 - analytical standards; W-082/S/LF
 - analytical samples before the analytical part; W-093/LF
 - analytical samples extracts until the end of the instrumental analysis; W-084/S/LF
- Centrifuge (> 3000 rpm); W-076/LF
- Cutter (knife grinder); W-133/LF, W-096/LF
- Polypropylene centrifuge tubes, with screw caps, 50 mL
- Plastic cups (stackable), 100 mL, for the storage of archived samples
- Volumetric flasks, 5 and 10 mL (class A)
- Automatic pipettes, 10 mL; WP-103/S/LF
- Laboratory syringes 10 µL, 50 µL, 100 µL, 250 µL, 500 µL and 1000 µL
- Vials with screw caps, amber glass, 10 mL
- Injection vials, 1.5 mL, amber glass, screw caps with septa butyl/PTFE
- Liquid Chromatography Instrument LC-MSMS Shimadzu 8050; WP-009/K/S/ko/LF;
- Syringe filters RC 0.22 µm, 25 mm

Identification of Instrumentation and Apparatus

Instrument settings:

Degazer DGU-403

Two pumps LC-40D XR

Autosampler SIL-40C XR

Column oven CTO-40S

Compressor, generator PEAK Genius 1051

HPLC Column – Hypercarb, 100 x 2.1 mm, 5 micron (Thermo Scientific); PN: 35005-102130;
LOT: 5-829

Pumps:

Mode – Binary gradient

Total Flow – 0.30 mL/min

- Mobile Phase A :1% acetic acid in water + 5% methanol

- Mobile Phase B: 1% acetic acid in methanol

Analytical procedure

Untreated samples for validation purposes were obtained from other ongoing studies.

Before the homogenization process, samples were stored frozen at < -18 °C. All the samples were homogenized at Test Facility, using a knife grinder. The homogenized specimens were further stored at ≤ -18 °C until beginning of analysis.

The extraction and analysis of validation samples was carried out on: 10.12.2021 (seed, plant), and 11.12.2021 (straw). 5 g (seed, plant)/ 2 g (straw) of the homogenized sample was weighed into a 50 mL

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centrifuge tube. 10 mL of water and 10 mL of acidified methanol (with 1 % of HCOOH) was added together with 50 µL of internal standard solution (IS WS I), and the mixture was shaken vigorously by hand for one minute, then centrifuged at 4700 rpm for 10 min for phase separation and finally subjected to a freezing process at $\leq -18^{\circ}\text{C}$ for 2 h. After that, extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis

Straw

2 g of the homogenized control sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added.

Plant, seed

5 g of the homogenized control sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added

Table C-1: Recovery results from confirmatory method validation of Triazole Derivative Metabolites (TAA, TA, 1,2,4-T, TLA) using the confirmatory analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Seed	1,2,4-T 70.20>43.05	0.010	91.9	2.12	-
	1,2,4-T 70.20>43.05	0.10	108.5	3.30	-
	1,2,4-T 70.20>42.05	0.010	86.3	8.56	-
	1,2,4-T 70.20>42.05	0.10	106.1	4.37	-
	TA 157.00>69.90	0.010	90.5	8.13	-
	TA 157.00>69.90	0.10	117.4	1.70	-
	TA 157.00>42.10	0.010	78.1	7.11	-
	TA 157.00>42.10	0.10	117.0	2.06	-
	TAA 128.00>69.90	0.010	98.7	1.94	-
	TAA 128.00>69.90	0.10	108.7	3.34	-
	TAA 128.00>43.10	0.010	105.5	3.61	-
	TAA 128.00>43.10	0.10	110.9	3.60	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	TLA 158.00>69.95	0.010	75.9	1.30	-
	TLA 158.00>69.95	0.10	109.1	2.27	-
	TLA 158.00>43.05	0.010	76.9	1.90	-
	TLA 158.00>43.05	0.10	107.4	3.04	-
Straw	1,2,4-T 70.20>43.05	0.010	113.2	2.66	-
	1,2,4-T 70.20>43.05	0.10	91.1	3.22	-
	1,2,4-T 70.20>42.05	0.010	100.5	14.0	-
	1,2,4-T 70.20>42.05	0.10	93.1	6.08	-
	TA 157.00>69.90	0.010	114.8	2.14	-
	TA 157.00>69.90	0.10	110.6	6.74	-
	TA 157.00>42.10	0.010	107.7	8.98	-
	TA 157.00>42.10	0.10	105.5	8.60	-
	TAA 128.00>69.90	0.010	93.1	4.99	-
	TAA 128.00>69.90	0.10	84.9	5.40	-
	TAA 128.00>43.10	0.010	92.1	11.7	-
	TAA 128.00>43.10	0.10	86.8	4.45	-
	TLA 158.00>69.95	0.010	115.4	3.00	-
	TLA 158.00>69.95	0.10	80.4	4.42	-
	TLA 158.00>43.05	0.010	115.5	3.36	-
	TLA 158.00>43.05	0.10	85.2	5.97	-
Plant	1,2,4-T 70.20>43.05	0.010	76.7	3.09	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	1,2,4-T 70.20>43.05	0.10	92.9	1.07	-
	1,2,4-T 70.20>42.05	0.010	76.7	3.92	-
	1,2,4-T 70.20>42.05	0.10	90.8	2.69	-
	TA 157.00>69.90	0.010	103.7	7.05	-
	TA 157.00>69.90	0.10	113.7	1.40	-
	TA 157.00>87.90	0.010	99.4	8.95	-
	TA 157.00>87.90	0.10	115.0	1.70	-
	TAA 128.00>69.90	0.010	81.7	11.6	-
	TAA 128.00>69.90	0.10	86.4	0.69	-
	TAA 128.00>43.10	0.010	104.8	10.5	-
	TAA 128.00>43.10	0.10	97.3	6.22	-
	TLA 158.00>69.95	0.010	78.2	2.38	-
	TLA 158.00>69.95	0.10	82.8	0.74	-
	TLA 158.00>43.05	0.010	103.3	3.89	-
	TLA 158.00>43.05	0.10	86.2	1.27	-

Table C-2: Methods suitable for the determination of the residues of Triazole Metabolites in Oilseed rape

	Residues
Author(s), year	S. Niewelt-Stasiak
Principle of method	LC MS/MS
Linearity (linear)	The linearity of the detector response was demonstrated by single determination of calibration standards at concentration levels ranging from 0.5 ppb (straw) or 1.00 ppb (plant, seed) to 500 ppb.

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	Residues
between mg/L) (correlation coefficient, expressed as r)	The coefficient of determination (R^2) were determined. Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration
Recovery	Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level. The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30\%$, and recovery at fortification level of 0.1 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20\%$
Accuracy Precision and repeatability	Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level. The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30\%$, and recovery at fortification level of 0.1 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20\%$
Specificity	LC-MS/MS method was used during the study. Two mass transitions were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for the matrix blank samples.
Assessment of matrix effects is presented	This effect was not observed during validation, but the method of preparing working calibration standards based on blank sample was used.
LOQ	The LOQ is the lowest validated fortification level for which an average recovery in the range of 70 – 120% (60 – 120 % in case of level ≤ 0.01 mg/kg) and $RSD \leq 20\%$ ($\leq 30\%$ in case of level ≤ 0.01 mg/kg) is achieved. For TDMs LOQ was successfully established at 0.010 mg/kg for oilseed rape (seed, straw, plant). LOD (limit of detection) was established at 0.004 mg/kg (seed, plant) and 0.005 mg/kg (straw). The limit of detection (LOD) was expressed as the lowest calibration standard
Comment	The analytical method for determining the residues of Triazole Derivative Metabolites (TDMs = 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid, Triazole-lactic acid) in oilseed rape (seed, straw and plant) meets the criteria of SANTE/2020/12830, Rev.2 guidelines document in terms of precision, accuracy and uncertainty

Conclusion

The results acquired during validation of the analytical method (accuracy and repeatability) were in the range of 70 – 120%. The limit of detection (LOD) was expressed as the lowest calibration standard and limit of quantification (LOQ) of the analytical method was established at 0.010 mg/kg for each of compounds (TAA, TA, 1,2,4-T, TLA) in oilseed rape. There were no interfering signals at retention time of analyzed compound in examined control matrix.

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Comments of zRMS	The purpose of this study was to validate an analytical method for the determination of residues of Triazole Derivative Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid) in sugar beet (roots, leaves). Specimen extraction and determination of residues of TDMs was performed using the QuPPe method. The specimens were prepared, extracted and analyzed following analytical procedures: - ANALYTICAL PROCEDURE DPL-60 Determination of residues of Triazole Derivative Metabolites (TAA, TA, 1,2,4-T, TLA) in food of plant origin using the QuPPe method and liquid chromatography technique with LC-MS / MS tandem mass spectrometry detection, based on: Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement, I. Food of Plant Origin (QuPPe-POMethod), Version 12 (22.07.2021) The limit of detection (LOD) in sugar beet (leaves and roots) was 0.002 mg/kg. The limit of quantification (LOQ) of the analytical method was 0.010 mg/kg for Triazole Derivative Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid) in sugar beet (roots, leaves). The total analytical procedure was performed and completed within 1 day in case of each matrix. For each analyte, two mass transitions were evaluated and used for quantification. Representative chromatograms were shown in the report. A third mass transition was monitored for confirmation of peak identity, but was not used for quantification. For TAA in sugar beet roots and TA in sugar beet leaves matrix effect is $< \pm 20\%$. In the rest of studied cases matrix effect calculated using equation exceed $\pm 20\%$. However to compensate matrix effects, the matrix -matched calibrations was used in case of each matrix. Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). For all analytes the mean recoveries were within 70 % – 120 %. The relative standard deviations over all testing intervals were $\leq 20\%$ for all analytes. The analytical method for determining the residues of Triazole Derivative Metabolites (TDMs: 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid, Triazole-lactic acid) in sugar beet (roots and leaves) meets the criteria of SANTE/2020/12830, Rev. 2 in terms of precision, accuracy and uncertainty. The study is accepted.
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Reference:

KCP 5.2/06

Report

Validation of an analytical method for the determination of residues of Triazole Derivative Metabolites (1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid) in sugar beet (roots, leaves), Validation Study No: VAL/21/2023

Guideline(s):

Regulation (EC) No 1107/2009
SANTE/2020/12830 Rev.2.

Deviations:

NO

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GLP: YES

Acceptability: YES

Materials and methods

The method for the determination of residues of TAA, TA, 1,2,4-T, TLA was validated in sugar beet (roots, leaves). The method was validated over the concentration range of 0.010-0.10 mg/kg ($\mu\text{g/g}$) with an estimated limit of detection at 0.002 mg/kg (sugar beet roots and leaves)

Reagents

- Deionized Water, Lot: 23H304043
- Methanol, CH₃OH, HPLC grade, Lot: 221115C007
- Formic Acid, HCOOH, > 98%, p.a. Lot: 22D114007
- Acetic Acid, CH₃COOH, > 98%, p.a., Lot: 19E024015

Equipment

- Electronic balance Class I; WP-011/W/S/LF
- Electronic balance Class II; WP-012/W/S/LF, WP-013/W/S/LF
- Freezers - storage of:
 - analytical standards; W-082/S/LF
 - analytical samples before the analytical part; W-093/LF
 - analytical samples extracts—freezing during extraction/preparation of samples; W-084/S/LF
 - analytical samples extracts until the end of the instrumental analysis; W-084/S/LF
 - archived analytical samples; W-083/LF (untreated), W-091/LF (treated)
- Centrifuge (> 3000 rpm); W-076/LF
- Cutter (knife grinder); W-133/LF, W-096/LF
- Polypropylene centrifuge tubes, with screw caps, 25 and 50 mL
- Plastic cups (stackable), 100 mL, for the storage of archived samples
- Volumetric flasks, 5 and 10 mL (class A)
- Automatic pipettes: 10–200 μL (WP-137/S/LF); 0.5-5.00 mL (WP-102/S/LF); 1.00-10.0 mL (WP-103/S/LF); 50-1000 μL (WP-138/S/LF); 0.1-1.00 mL (WP-101/S/LF)
- Laboratory syringes: 10 μL
- Vials with screw caps, amber glass, 10 mL
- Injection vials, 2.5 mL, amber glass, screw caps with septa butyl/PTFE
- Syringe filters RC 0.22 μm , 25 mm
- Liquid Chromatography Instrument LC-MS/MS Sciex Qtrap 6500+ with SelexION+
; WP-144/K/S/ko/LF
- Chromatographic column: Hypercarb, 100 x 2.1 mm, 5 micron (Thermo Scientific), Lot: 5-1079

Identification of Instrumentation and Apparatus

Instrument settings:

Degazer DGU-405
Two pumps LC-40D XR
Autosampler SIL-40C XR
Column oven CTO-40C

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Compressor, generator PEAK Genius 1024
HPLC Column – Hypercarb, 100 x 2.1 mm, 5 micron (Thermo Scientific), Lot: 5-1079

Pumps:

Mode – Binary gradient

Total Flow – 0.60 mL/min

- Mobile Phase A: 1% acetic acid in water + 5% methanol

- Mobile Phase B: 1% acetic acid in methanol

Analytical procedure

Untreated samples for validation purposes were obtained from other ongoing studies. Before the homogenization process, samples were stored frozen at $< -18^{\circ}\text{C}$. All the samples were homogenized at Test Facility, using a knife grinder. The homogenized specimens were further stored at $\leq -18^{\circ}\text{C}$ until beginning of analysis.

The extraction and analysis of validation samples was carried out on: - 24.11.2023: sugar beet (roots) - 28.11.2023: sugar beet (leaves) 5 g (leaves)/ 10 g (roots) of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of water (in case of leaves samples) and 10 mL of acidified methanol (with 1 % of HCOOH) was added together with 50 μL of internal standard solution (IS WS I), and the mixture was shaken vigorously by hand for one minute, then centrifuged at 4700rpm for 10 min for phase separation and finally subjected to a freezing process at $\leq -18^{\circ}\text{C}$ for 2 h. After that, extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis.

Fortification samples: For analytical sequence two samples blank matrix, two samples at limit of detection (LOD), five procedural recoveries at the level of LOQ and five at the level 10 x LOQ were prepared. 5 g (leaves)/ 10 g (roots) of the homogenized untreated sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added (see Table 2 and 3 for amounts and section 3.2.3 for information on solution preparation), and the sample was extracted as described above.

Table D-1: Recovery results from confirmatory method validation of Triazole Derivative Metabolites (TAA, TA, 1,2,4-T, TLA) using the confirmatory analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Sugar beet (roots)	1,2,4-T 70.00 → 70.00	0.010	94.0	1.71	-
	1,2,4-T 70.00 → 70.00	0.10	93.8	1.03	-
	1,2,4-T 70.00 → 43.10	0.010	92.8	3.21	-
	1,2,4-T 70.00 → 43.10	0.10	94.4	2.31	-
	TA 157.00 → 70.00	0.010	88.7	3.21	-
	TA 157.00 → 70.00	0.10	88.3	0.82	-
	TA 157.00 → 88.10	0.010	85.9	2.51	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	TA 157.00→ 88.10	0.10	87.4	0.89	-
	TAA 128.00→ 70.00	0.10	92.7	1.34	-
	TAA 128.00→ 70.00	0.010	94.5	1.01	-
	TAA 128.00→ 43.10	0.10	89.0	3.30	-
	TAA 128.00→ 43.10	0.010	92.7	1.44	-
	TLA 158.00→ 70.00	0.10	87.4	1.53	-
	TLA 158.00→ 70.00	0.010	91.8	0.34	-
	TLA 158.00→ 112.00	0.10	87.2	1.68	-
	TLA 158.00→ 112.00	0.010	87.5	0.83	-
	TLA 158.00→ 112.00	0.010	87.5	0.83	-
Sugar beet (leaves)	1,2,4-T 70.00→ 70.00	0.10	98.0	6.23	-
	1,2,4-T 70.00→ 70.00	0.010	95.6	4.88	-
	1,2,4-T 70.00→ 43.10	0.10	100.0	9.84	-
	1,2,4-T 70.00→ 43.10	0.010	96.1	4.14	-
	TA 157.00→ 70.00	0.10	91.7	3.57	-
	TA 157.00→ 70.00	0.010	94.9	4.70	-
	TA 157.00→ 88.10	0.10	91.1	1.59	-
	TA 157.00→ 88.10	0.010	96.6	4.28	-
	TAA 128.00→ 70.00	0.10	94.7	5.86	-
	TAA 128.00→ 70.00	0.010	91.9	4.34	-
	TAA 128.00→ 43.10	0.10	93.2	8.68	-
	TAA 128.00→ 43.10	0.010	92.9	2.55	-
	TLA 158.00→ 70.00	0.10	92.6	5.80	-
	TLA 158.00→ 70.00	0.010	92.6	5.80	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	158.00→ 70.00				
	TLA 158.00→ 70.00	0.010	93.4	4.55	-
	TLA 158.00→ 112.00	0.10	95.8	6.44	-
	TLA 158.00→ 112.00	0.010	92.3	4.41	-

Table D-2: Methods suitable for the determination of the residues of residues of Triazole Metabolites in Sugar beet

	Residues
Author(s), year	S. Niewelt-Stasiak
Principle of method	LC MS/MS
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	The linearity of the detector response was demonstrated by single determination of calibration standards at concentration levels ranging from 0.5 ppb (leaves) or 1 ppb (roots) to 500 ppb. The coefficient of determination (R^2) were determined. Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration.
Recovery	Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level. The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30\%$, and recovery at fortification level of 0.1 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20\%$
Accuracy Precision and repeatability	Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level. The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30\%$, and recovery at fortification level of 0.1 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20\%$
Specificity	LC-MS/MS method was used during the study. Two mass transitions were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for the matrix blank samples.
Assessment of matrix effects is presented	For TAA in sugar beet roots and TA in sugar beet leaves matrix effect is $< \pm 20\%$. In the rest of studied cases matrix effect calculated using equation exceed $\pm 20\%$.
LOQ	The LOQ is the lowest validated fortification level for which an average recovery in the range of 70 – 120% (60 – 120 % in case of level ≤ 0.01 mg/kg) and $RSD \leq 20\%$ ($\leq 30\%$ in case of level ≤ 0.01 mg/kg) is achieved. For TDMs LOQ was successfully established at 0.010 mg/kg for sugar beet (roots, leaves). LOD (limit of detection) was established at 0.002 mg/kg (roots, leaves). The limit of

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	Residues
	detection (LOD) was expressed as the lowest calibration standard.
Comment	The analytical method for determining the residues of Triazole Derivative Metabolites (TDMs: 1,2,4-Triazole, Triazole-alanine, Triazole-acetic acid, Triazole-lactic acid) in sugar beet (roots and leaves) meets the criteria of SANTE/2020/12830, Rev. 2, 14 February 2023 guidelines document in terms of precision, accuracy and uncertainty. The method was validated over the concentration range of 0.01 - 0.10 mg/kg (µg/g) for triazole derivative metabolites.

Conclusion

The method was validated over the concentration range of 0.01 - 0.10 mg/kg (µg/g) for triazole derivative metabolites. The limit of detection (LOD) that was expressed as the lowest calibration standard was established at 0.002 mg/kg in sugar beet (roots and leaves). Limit of quantification (LOQ) of the analytical method was established at 0.010 mg/kg for each of compounds (TAA, TA, 1,2,4-T, TLA) in sugar beet (roots and leaves).

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

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

No new or additional studies have been submitted

A 2.1.2.1.1. Analytical method 1

A 2.1.2.1.1.1 Method validation

No new or additional studies have been submitted.

A 2.1.2.1.1.2 Independent laboratory validation

No new or additional studies have been submitted.

A 2.1.2.1.1.3 Confirmatory method.

No confirmatory method is required.

A 2.1.2.1.1.4 Extraction efficiency

No new or additional studies have been submitted.

A 2.1.2.1.2 Analytical method 2

A 2.1.2.1.3 Extraction efficiency

No new or additional studies have been submitted.

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

~~No new or additional studies have been submitted~~

A 2.1.2.2.1 Analytical method 1

A 2.1.2.2.1.1 Method validation

~~No new or additional studies have been submitted.~~

Comments of zRMS	Sample extraction and determination of analyte levels was performed according to the QuPpe-PO-Method. Quantification was performed by use of LC-DMS-MS/MS detection with isotopically labelled internal standards. The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte with a limit of detection (LOD) set at 0.003 mg/kg (30 % of the LOQ). The fortification level for storage samples was at ten times the limit of quantification (LOQ) of the method (0.1 mg/kg) with all analytes fortified
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	separately on aliquots of homogenised control sample material. Storage samples were kept at $\leq -18^{\circ}\text{C}$. Example chromatograms for each analyte representing at least control samples and samples fortified at 10x LOQ level were included in the report. For triazole metabolites 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid, the mean recoveries over all testing intervals were within 85 % – 93 % with RSD $<20\%$ for honey. The study is deemed sufficient for assessing the stability of triazole metabolites 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in honey upon storage at $\leq -18^{\circ}\text{C}$ for 15 months (459 days). The study is accepted.
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Reference: KCP 5.2/07

Report Storage Stability of Triazole Metabolites in Honey under Deep Frozen Conditions
Study no: S21-05867

Guideline(s): Regulations (EU) 283/2013 and 284/2013 implementing Regulation (EC) 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC OECD 506, 2007 (OECD guideline for the testing of chemicals / Stability of pesticide residues in stored commodities) 7032/VI/95, rev. 5 (EC guideline, Appendix H of working document 1607/VI/97, rev. 2, June 1999 – Storage Stability of Residue Samples) SANTE/2020/12830, rev.1

Deviations: NO

GLP: YES

Acceptability: YES

Materials and methods

The method is based on the QuPpe-PO-Method: “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC or IC MS/MS Measurement”.

Reagents

- Formic acid (Riedel de Haen, Art. No. 33015)
- Methanol (Chromasolv, Sigma-Aldrich, Art. No. 34860)
- Water (VWR, HiPerSolv CHROMANORM for HPLC, Art. No. 23595.328)

Instrumentation and Apparatus

- ☐ Adjustable pipettes
 - (Reference 10-100 μL and Reference, 100-1000 μL , Eppendorf and HandyStep with 12.5 mL tip,
 - Brand)
 - Common laboratory glassware
 - Centrifuge tubes with caps, PP, 50 mL

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- (Sarstedt AG & Co., Art. No. 62547254)
- Centrifuge tubes with caps, PP, 15 mL
- (Sarstedt AG & Co., Art. No. 62554502)
- HPLC Autosampler vials, 1.8 mL
- Laboratory centrifuge for 50 mL centrifuge tubes
- (Rotina Hettich 420R, Andreas Hettich GmbH & Co.KG)
- Nitrogen Evaporator
- (N-EVAP, Organomation Associates, Inc., Art. No. 11155-RT)
- Platform shaker
- (SM 30 C, Edmund Bühler GmbH, Art. No. 6105 000)
- Test tubes, with ground stoppers and graduation marks at 2.5, 5.0 and 10 mL
- Volumetric pipettes, e.g. 1, 2 and 5 mL
- Vortex mixer
- (Reax TOP, HEIDOLPH Instruments GmbH & Co. KG, Art. No. 541-10000-00-1)
- Water bath
- (linear shaking water bath, GFL GmbH, Art. No. 1083)
- Single use filter, 0.45 µm
- (Minisart High Flow PES, VWR, Art. No. 516-0901)

Summary of chromatographic and mass spectrometric conditions

Detection Method 1: Chromatographic conditions for the quantification of 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey (incl. confirmation of TA)				
UPLC system	1290 Infinity Binary LC System, Agilent Technologies (UHPLC, ≤ 1200 bar)			
Pre-column	Not used			
Column	Hypercarb (100 mm x 3 mm, 5 µm) Thermo Scientific, Art. No. 35005-103030			
Column oven temperature	60 °C			
Injection volume	25 µL			
Mobile phases	Eluent A: Methanol containing 0.5 % (v/v) formic acid Eluent B: Water containing 0.5 % (v/v) formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0	0	100	600
	3.0	0	100	600
	5.0	50	50	600
	8.0	50	50	600
	8.1	0	100	600
	11.0	0	100	600
Divert valve	to mass spectrometer from 1.0 min to 7.0 min			
Retention time(s)	1,2,4-Triazole (T): approx. 1.6 min Triaizole alanine (TA): approx. 2.3 min Triaizole acetic acid (TAA): approx. 5.2 min Triaizole lactic acid (TLA): approx. 5.7 min			

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Detection Method 1: Mass spectrometric conditions for the quantification of 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey (incl. confirmation of TA)									
MS system	SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupole mass spectrometer with device for ion mobility spectrometry (SelexION))								
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)								
Polarity	Positive ion mode								
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)								
Capillary voltage (IS)	4000 V (pos)	Ionspray turbo heater (TEM)		350 °C					
Curtain gas (CUR)	Nitrogen set at 35 (arbitrary units)	Gas flow 1 (GS1)		Zero-grade air set at 45 (arbitrary units)					
Collision gas (CAD)	Nitrogen set at 7 (arbitrary units)	Gas flow 2 (GS2)		Zero-grade air set at 65 (arbitrary units)					
DMS Modifier (MD)	None	DMS Temperature		Low					
DMS Resolution Enhancement (DR)	Open	Scheduled MRM Window		120 sec					
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Separation voltage# (SV) [V]	Compensation Voltage# (COV) [V]	DMS Offset (DMO) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Retention Time [min]
1,2,4-Triazole (T)	70 → 43 [#]	140	3000	-15.5	25	10	30	15	1.6
	75 → 46 [*]	140	3000	-15.5	25	10	30	15	1.6
Triazole acetic acid (TAA)	128 → 70 [#]	100	3000	-5.5	15	10	25	15	5.9
	133 → 75 [*]	100	3000	-5.5	15	10	25	15	5.9
Triazole lactic acid (TLA)	158 → 70 [#]	90	3500	-2.0	15	10	25	15	5.8
	163 → 75 [*]	90	3500	-2.0	15	10	25	15	5.8
Triazole alanine (TA)	157 → 70 [#]	90	3000	-2.0	15	10	20	15	2.3
	162 → 75 [*]	90	3000	-2.0	15	10	20	15	2.3

Table E-1: Recovery results from confirmatory method validation of Triazole Metabolites using the confirmatory analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Honey	1,2,4-Triazole	0.1	93	9.1	!
	Triazole Alanine	0.1	87	6.3	!
	Triazole Acetic Acid	0.1	89	5.2	!
	Triazole Lactic Acid	0.1	85	7.1	!

Table E-2: Methods suitable for the determination of the residues of residues of Triazole Metabolites in Honey

	Residues
Author(s), year	Sebastian Mahlow, Kelly Okeoghene Ereretevwe, 2023

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	Residues																																																																		
Principle of method	LC-DMS-MS/MS detection with isotopically labelled internal standards																																																																		
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	The linearity of the detection system was demonstrated by use of standard solutions covering a working range which was equivalent no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level in any sample extract The coefficients of determination (R2) were ≥ 0.99.																																																																		
Recovery	<table><tr><th rowspan="2">Matrix</th><th rowspan="2">Fortification level (mg/kg)</th><th colspan="3">Recovery (%)</th><th rowspan="2">Mean (%)</th><th rowspan="2">Rel. Std. Dev. (%)</th></tr><tr><th>Day 0 Testing</th><th>3 Months Testing</th><th>15 Months Testing</th></tr><tr><td colspan="7">1,2,4-Triazole</td></tr><tr><td>Honey</td><td>0.1</td><td>82, 92, 90</td><td>109, 97</td><td>89, 88</td><td>93</td><td>9.1</td></tr><tr><td colspan="7">Triazole Alanine</td></tr><tr><td>Honey</td><td>0.1</td><td>88, 85, 85</td><td>80, 84</td><td>97, 92</td><td>87</td><td>6.3</td></tr><tr><td colspan="7">Triazole Acetic Acid</td></tr><tr><td>Honey</td><td>0.1</td><td>93, 93, 90</td><td>84, 83</td><td>95, 87</td><td>89</td><td>5.2</td></tr><tr><td colspan="7">Triazole Lactic Acid</td></tr><tr><td>Honey</td><td>0.1</td><td>82, 78, 80</td><td>94, 91</td><td>90, 83</td><td>85</td><td>7.1</td></tr></table> No residue above 30 % of the LOQ level was detected in any control sample extract Recoveries are without any blank correction	Matrix	Fortification level (mg/kg)	Recovery (%)			Mean (%)	Rel. Std. Dev. (%)	Day 0 Testing	3 Months Testing	15 Months Testing	1,2,4-Triazole							Honey	0.1	82, 92, 90	109, 97	89, 88	93	9.1	Triazole Alanine							Honey	0.1	88, 85, 85	80, 84	97, 92	87	6.3	Triazole Acetic Acid							Honey	0.1	93, 93, 90	84, 83	95, 87	89	5.2	Triazole Lactic Acid							Honey	0.1	82, 78, 80	94, 91	90, 83	85	7.1
Matrix	Fortification level (mg/kg)			Recovery (%)					Mean (%)	Rel. Std. Dev. (%)																																																									
		Day 0 Testing	3 Months Testing	15 Months Testing																																																															
1,2,4-Triazole																																																																			
Honey	0.1	82, 92, 90	109, 97	89, 88	93	9.1																																																													
Triazole Alanine																																																																			
Honey	0.1	88, 85, 85	80, 84	97, 92	87	6.3																																																													
Triazole Acetic Acid																																																																			
Honey	0.1	93, 93, 90	84, 83	95, 87	89	5.2																																																													
Triazole Lactic Acid																																																																			
Honey	0.1	82, 78, 80	94, 91	90, 83	85	7.1																																																													
Accuracy Precision and repeatability	For triazole metabolites 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid, the mean recovery for samples extracted without any storage (i.e. day 0 storage samples and concurrent recoveries) were 93%, 87%, 89% and 85%, respectively. Relative standard deviations were ≤ 20 % for all analytes. For duplicate analysis of storage samples from the same time point the difference between the highest and lowest recovery did not exceed 20 %. These values demonstrate satisfying analytical performance for all analytes and matrix while analysing the storage samples																																																																		
Specificity	The control samples showed no significant interference (above 30 % of LOQ) at the retention time of the analyte(s) in any investigated matrix, therefore showing that the method is highly specific. Blank correction was not performed.																																																																		
Assessment of matrix effects is presented	Referring to the requirements of SANTE/2020/12830 Rev.2 if the matrix effect exceeds 20% LOQ, the "matrix-matched" calibration should be introduced. The highest and lowest recovery did not exceed 20 %.																																																																		
LOQ	The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte with a limit of detection (LOD) set at 0.003 mg/kg (30 % of the LOQ).																																																																		
Comment	The method was successfully validated for determination of all analytes with an LOQ of 0.01 mg/kg according to the guidance document(s) SANTE/2020/12830, Rev.2																																																																		

Conclusion

The method was successfully validated for determination of all analytes with an LOQ of 0.01 mg/kg according to the guidance document(s) SANTE/2020/12830, Rev.2 With regard to selectivity, accuracy and precision, the analytical methods were applied successfully.

The study is deemed sufficient for assessing the stability of triazole metabolites 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in honey upon storage at ≤ -18 °C for 15 months.

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A 2.1.2.2.1.2 Independent laboratory validation.

~~No new or additional studies have been submitted.~~

Comments of zRMS	The study is accepted.
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Reference:

KCP 5.2/08

Report

Independent Laboratory Validation of Multi-Residue Method QuEChERS for Determination of Prothioconazole, Prothioconazole-desthio and Azoxystrobin in Honey and Pollen and QuPpe-PO-Method for Determination of 1,2,4 Triazole (T), Triazole Alanine (TA), Triazole Acetic Acid (TAA) and Triazole Lactic Acid (TLA) in Honey, Study no: S23-104559

Guideline(s):

Regulations (EU) 283/2013 and 284/2013 implementing Regulation (EC) 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC SANTE/2020/12830, rev.2 for risk assessment and/or monitoring

Deviations:

NO

GLP:

YES

Acceptability:

YES

Materials and methods

To independently validate (ILV) the multi-residue method QuEChERS for the determination of prothioconazole, prothioconazole-desthio and azoxystrobin in multiflower honey and pollen and QuPpe-PO-Method for determination of triazole derivative metabolites (TDMs) 1,2,4 triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in multiflower honey in accordance to guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reagents

- Acetonitrile, HPLC grade (VWR)
- Millipore water (supply at test facility)
- Methanol and water, LC-MS grade (Merck)
- Magnesium Sulfate (anhydrous), Acros Organics
- Sodium Chloride, Sigma-Aldrich
- Ammonium Formate, Sigma-Aldrich
- PTFE Syringe Filter, 0.45 µm, Macherey Nage

Instrumentation and Apparatus

- Analytical Balance ED2202S-CW, Sartorius
- Analytical Balance XS205DU, Mettler-Toledo

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- Knife Mill GM300, Retsch
- Vortex mixer, Reax TOP, Heidolph
- Ultrasonic bath, Transsonic 700, Elma Hans Schmidbauer
- Mechanical horizontal shaker HS250B, IKA
- Laboratory centrifuge Rotanta 460, Hettich
- Various adjustable pipettes
- HPLC Autosampler vials
- Common laboratory glassware

Summary of chromatographic and mass spectrometric conditions

Detection Method 1: Chromatographic conditions for the quantification of 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey (incl. confirmation of TA)				
HPLC system	Agilent 1290 Infinity II Binary LC System, HTS-xt autosampler, MayLab MistraSwitch column oven			
Pre-column	Phenomenex AJ0-4287, 4x3 mm C ₁₈			
Column	Thermo Hypercarb (100 mm x 4.6 mm, 5 µm, Part No. 35005-104630)			
Column oven temperature	60 °C			
Injection volume	25 µL			
Mobile phases	Eluent A: Water containing 0.5 % (v/v) formic acid Eluent B: Methanol containing 0.5 % (v/v) formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.0	100	0	600
	3.0	100	0	600
	5.0	50	50	600
	8.0	50	50	600
	8.1	100	0	600
	11.0	100	0	600
Divert valve	to mass-spectrometer from 0 min to 11.0 min			
Retention time(s)	1,2,4-Triazole (T): approx. 3.7 min Triazole alanine (TA): approx. 5.0 min Triazole acetic acid (TAA): approx. 8.0 min Triazole lactic acid (TLA): approx. 7.8 min			

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Detection Method 1: Mass spectrometric conditions for the quantification of 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in honey (incl. confirmation of TA)									
MS system	SCIEX TripleQuad 6500+ System, SCIEX (Triple quadrupole mass spectrometer with device for ion mobility spectrometry (SelexION))								
Ionisation type	Electrospray ionisation (ESI, TurbolonSpray)								
Polarity	Positive ion mode								
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)								
Capillary voltage (IS)	4000 V (pos)	Ionspray turbo heater (TEM)		350 °C					
Curtain gas (CUR)	35 (arbitrary units)	Gas flow 1 (GS1)		60 (arbitrary units)					
Collision gas (CAD)	10 (arbitrary units)	Gas flow 2 (GS2)		60 (arbitrary units)					
DMS Modifier (MD)	None	DMS Temperature (DT)		Low					
DMS Resolution Enhancement (DR)	Open	Scheduled MRM		No					
Analyte monitored	Mass transition monitored (m/z)	Declustering potential (DP) [V]	Separation voltage ^{##} (SV) [V]	Compensation Voltage ^{##} (COV) [V]	DMS Offset (DMO) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell Time [min]
1,2,4-Triazole (T)	70 → 43 [#]	101	2200	-9.0	-9.4	10	31	20	125
	75 → 46 [*]	101	2200	-9.0	-9.4	10	31	20	125
Triazole acetic acid (TAA)	128 → 70 [#]	46	3200	-5.75	-10	10	25	8	60
	133 → 75 [*]	46	3200	-5.75	-10	10	25	8	60
Triazole lactic acid (TLA)	158 → 70 [#]	46	2800	-2.00	-9.7	10	25	10	60
	163 → 75 [*]	46	2800	-2.00	-9.7	10	25	10	60
Triazole alanine (TA)	157 → 70 [#]	61	1400	0.25	-9.8	10	19	8	125
	157 → 88 [†]	61	1400	0.25	-9.8	10	17	6	125
	162 → 75 [*]	61	1400	0.25	-9.8	10	19	8	60

[#] proposed and used for quantification.

[†] used for confirmation. Both of the mass transitions listed can be used for quantification.

^{*} The monitored mass transition corresponds to the associated internal standards.

^{##} COV values may need to be adjusted on occasion to yield optimal selectivity and sensitivity. If results comparable to the results shown in this report cannot be obtained, SV values might also require adjustment.

Table F-1: Recovery results from confirmatory method validation of Azoxystrobin and Triazole Metabolites using the confirmatory analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Honey	1,2,4-Triazole 70→43 Detection Method Nr. 1 [#]	0.01	94	3.3	-
	1,2,4-Triazole 70→43 Detection Method Nr. 1 [#]	0.1	86	2.2	-
	1,2,4-Triazole 70→43 Detection Method Nr. 2 ^{##}	0.01	85	7.4	-
	1,2,4-Triazole 70→43 Detection	0.1	87	2.3	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	Method Nr. 2##				
	Triazole alanine 157→70 Detection Method Nr. 1	0.01	104	5.3	-
	Triazole alanine 157→70 Detection Method Nr. 1	0.1	92	2.0	-
	Triazole alanine 157→88 Detection Method Nr. 1	0.01**	99 (123)	6.0 (4.8)	-
	Triazole alanine 157→88 Detection Method Nr. 1	0.1**	83 (85)	4.3 (4.2)	-
	Triazole acetic acid 128→70 Detection Method Nr. 1#	0.01	83	3.4	-
	Triazole acetic acid 128→70 Detection Method Nr. 1#	0.1	80	3.7	-
	Triazole acetic acid 128→70 Detection Method Nr. 2#	0.01	90	3.2	-
	Triazole acetic acid 128→70 Detection Method Nr. 2#	0.1	81	2.7	-
	Triazole lactic acid 158→70 Detection Method Nr. 1#	0.01	98	2.3	-
	Triazole lactic acid 158→70 Detection Method Nr. 1#	0.1	93	2.2	-

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Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	Triazole lactic acid 158→70 Detection Method Nr. 2#	0.01	86 (106)	5.2 (4.2)	-
	Triazole lactic acid 158→70 Detection Method Nr. 2#	0.1	86 (88)	3.7 (3.6)	-

No residue above 30 % of the LOQ level was detected in any control sample extract. Exact fortification levels are 0.0096 and 0.096 mg/kg. Fortification levels are given rounded in the table.

*Mean of double injection

***Recoveries are corrected for corresponding mean control residue (0.0023 mg/kg) (uncorrected values in brackets) ***Recoveries are corrected for corresponding mean control residue (0.0020 mg/kg) (uncorrected values in brackets)

For corresponding mean control residue (0.0020 mg/kg) (uncorrected values in brackets)
 All other recoveries are without any blank correction. Analyte: Final determination as: Residues calculated as: 1,2,4-Triazole 1,2,4-Triazole 1,2,4-Triazole Triazole alanine Triazole alanine Triazole alanine Triazole acetic acid Triazole acetic acid Triazole acetic acid Triazole lactic acid Triazole lactic acid Triazole lactic acid

Table F-2: Methods suitable for the determination of the residues of residues of Azoxystrobin and Triazole Metabolites in Honey

	Residues									
Author(s), year	Diana Kreidler, 2024									
Principle of method	LC-MS/MS									
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	Method 1: The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at seven (7) concentration levels ranging from 0.7 ng/mL to 30 ng/mL. This range corresponds to a mass fraction level of 0.0028 mg/kg to 0.12 mg/kg and thus covers the range from no more than 28 % of the limit of quantification (LOQ) and at least +20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Correlation coefficients (R) were ≥ 0.99. Method 2: The linearity of the detector response was demonstrated by single determination of solvent calibration standards at seven (7) concentration levels ranging from 0.3 ng/mL to 30 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.3 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least +20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Correlation coefficients (R) were ≥ 0.99.									
Recovery	Mass Transition	Matrix	Fortification level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)	

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	Residues								
	1,2,4-Triazole								
	m/z 70→43 Detection Method Nr. 1 [#]	Honey	0.01	98*, 96, 90, 92, 92	94	3.3	5	90	5.2
			0.1	87*, 84, 85, 85, 89	86	2.2	5		
	m/z 70→43 Detection Method Nr. 2 ^{##}		0.01	95*, 88, 80, 84, 79	85	7.4	5	86	5.2
			0.1	85, 90, 86, 88, 87	87	2.3	5		
	Triazole alanine								
	m/z 157→70 [#] Detection Method Nr. 1	Honey	0.01	101*, 111, 100, 99, 108	104	5.3	5	98	7.7
			0.1	93*, 92, 91, 89, 94	92	2.0	5		
	m/z 157→88 ^{##} Detection Method Nr. 1		0.01**	96*(120), 97 (121), 108 (132), 93 (117), 101(125)	99 (123)	6.0 (4.8)	5	91 (104)	10.8 (19.8)
			0.1**	82* (85), 81 (84), 81 (83), 80 (82), 89 (91)	83 (85)	4.3 (4.2)	5		
	Triazole acetic acid								
	m/z 128→70 Detection Method Nr. 1 [#]	Honey	0.01	83*, 79, 86, 84, 85	83	3.4	5	82	3.9
			0.1	81*, 82, 77, 78, 84	80	3.7	5		
	m/z 128→70 Detection Method Nr. 2 ^{##}		0.01	94*, 88, 88, 87, 91	90	3.2	5	85	6.0
			0.1	83*, 81, 81, 78, 84	81	2.7	5		
	Triazole lactic acid								
m/z 158→70 Detection Method Nr. 1 [#]	Honey	0.01	99*, 95, 98, 97, 101	98	2.3	5	95	3.6	
		0.1	93*, 94, 91, 95, 90	93	2.2	5			
m/z 158→70 Detection Method Nr. 2 ^{##}		0.01***	82* (103), 92 (113), 85 (105), 89 (109), 82 (102)	86 (106)	5.2 (4.2)	5	86 (97)	4.2 (10.5)	
		0.1***	84* (86), 89 (91), 90 (92), 82 (84), 87 (89)	86 (88)	3.7 (3.6)	5			
Accuracy Precision and repeatability	Five (5) fortifications at 0.01 mg/kg (LOQ) (unrounded 0.0096 mg/kg for method 2) Five (5) fortifications at 0.1 mg/kg (10x LOQ) (unrounded 0.096 mg/kg for method 2), analytes fortified jointly. Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2: Concentration Level (mg/kg) Range of Mean Recoveries (%) Precision, Rel. Std. Dev. (%) ≤ 0.01 60 - 120 ≤ 30 > 0.01 - ≤ 0.1 70 - 120 ≤ 2								
Specificity	Demonstrated by validation of two (2) mass transitions or two HPLC methods. Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ with except for pollen blank sample for azoxystrobin and prothioconazole-desthio with residues < LOQ.								
Assessment of matrix effects is presented	Significant for all analytes in matrix pollen and for prothioconazol in matrix honey (≥ 20 %), insignificant for all other analytes in matrix honey (< 20 %). (Method 1) Isotopically labelled internal standards were used for quantification so that possible matrix effects on determination are automatically accounted for when using the response ratio of analyte and internal standard for quantification. Therefore, matrix effects on detection were not determined. (Method 2								
LOQ	0.01 mg/kg for all analytes (in absolute terms) (lowest validated fortification level) (Method 1 and Method 2)								
Comment	The methods were found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring								

Conclusion

Both methods were successfully independently validated for the determination of azoxystrobin in matrices honey and pollen, as well as for the determination of 1,2,4 triazole (T), triazole alanine (TA),

triazole acetic acid (TAA) and triazole lactic acid (TLA) in matrix honey from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg (absolute terms) according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

A 2.1.2.2.1.3 Confirmatory method (if required)

No confirmatory method is required.

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

No new or additional studies have been submitted

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

A 2.1.2.7 Other Studies/ Information

No new or additional studies have been submitted.