FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: **SNS-F-11**

Product names: **DISFERA 90 EC/ LIPOSTAR 90 EC**

Chemical active substance:

Difenoconazole, 90 g/L

Central Zone

Zonal Rapporteur Member State: **Poland**

CORE ASSESSMENT Poland

(authorization)

Applicant: **Synthos Agro Sp. z o. o.**

Submission date: **01/2024**

MS Finalisation date:**07/2024; 10/2024; 11/2024**

Version history

When	What
01/2024	Initial dRR
05/2024	Addidion of analytical methods supporting ecotoxicological studies.
07/2024	zRMS evaluation
10/2024	Final assessment after commenting period
11/2024	The final RR after the second round of commenting

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5 Analytical methods

5.1 Conclusion and summary of assessment

Submitted data are sufficient for evaluation of a method analysis used to determine the active substance and relevant impurity concentration in PPP. There are no data gaps. Analytical method (RP-HPLC/DAD) for determination of active substances Difenoconazole and HS-GC-FID method for determination relevant impurity toluene in plant protection product has been validated and meet criteria of specificity, linearity, precision and accuracy according to the requirement SANCO 3030/99 rev. 5, therefore it is acceptable.

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
Cereals	Supported
Oilseeds	Supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Reference:	Validation included in the following reports:
Report	Jarosław Kupiec, M.Sc., 2022/ Synthos Agro Validation included in the report: SNS-F-11 Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The determination of the active ingredient – difenoconazole content in SNS-F-11 preparation were carried

out in accordance with the method – MT/BA-05/23 – developed and validated according to EU requirements described in SANCO/3030/99 rev. 5 (22/03/19) guideline.
The method (MT/BA-05/23) is based on determination of difenoconazole using reversed phase high performance liquid chromatography (RP-HPLC) with DAD detection at wavelength 206 nm and external standard at the initial stage and after accelerated storage.

Examined material

Examined material:	SNS-F-11
Date of production:	03.2023
Batch number:	S/1/090323
Manufacturer:	Synthos Agro Sp. z o.o.

Reference material

Difenoconazole, IPO 930, batch 3A/22, purity 99.80%, storage at room temperature Validation - Results and discussions

Apparatus and materials

- Shimadzu liquid chromatograph equipped with DAD detector (WPiB: BA-P/15-S)
- Column: Kinetex Biphenyl, 250 x 4.6 mm, 5µm
- Analytical balance Mettler Toledo XS 205 DU/M, accuracy 0.01 mg
- Ultrasonic bath
- Volumetric flasks
- Automatic pipette BRAND
- Syringes
- Syringe filters Pureland PTFE 0.22 µm

Reagents

- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC-Super Gradient, POCh
- analytical standard – Difenoconazole (IPO 930)

Preparation of solutions

Standards solutions

About 10 mg of Difenoconazole standard was weighed (with the accuracy of 0.01 mg) into two separate 10 ml flasks with a screw cap and acetonitrile was added up to the volume. The flasks were put into the ultrasonic bath for 5 min. After cooling, solutions were diluted and analyzed.

Difenoconazole – standard, purity = 99.80%				
Chromatogram name	Mass [mg]	C’ [mg/ml]	V [ml]	C’’ [mg/ml]
std1	9.54	0.9521	0.70	0.1333
std2	10.17	1.0150	1.20	0.2436

Specimen solutions

About 22 mg of examined specimen was weighed (with the accuracy of 0.01 mg) into a 10 ml flask with a screw cap. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath for 5 min. After cooling, acetonitrile was added up to the volume and solution was filtered and analyzed.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance difenoconazole in plant protection product SNS-F-11

	Difenoconazole
Author(s), year	Jarosław Kupiec, M.Sc., 2023
Principle of method	The determination of the active ingredient – difenoconazole content in SNS-F-11 preparation were carried out in accordance with the method – MT/BA-05/23 – developed and validated according to EU requirements described in SANCO/3030/99 rev. 5 (22/03/19) guideline. The method (MT/BA-05/23) is based on determination of difenoconazole using reversed phase high performance liquid chromatography (RP-HPLC) with DAD detection at wavelength 206 nm and external standard at the initial stage and after accelerated storage. Chromatographic conditions: Shimadzu liquid chromatograph equipped with DAD detector (WPiB: BA-P/15-S) Column: Kinetex Biphenyl, 250 x 4.6 mm, 5µm Oven temperature: 30 ºC Flow rate: 1.0 ml/min Wavelength λ = 206 nm Volume injection: 5 µl Mobile phase composition: acetonitrile + water (50 + 50, v/v) Under the above conditions the retention time of Difenoconazole is about 14.3 ± 0.2 min and the total time of analysis is 30.0 min.
Linearity **Linear between:** **0.1027 mg/ml to 0.3081 mg/ml** **(approximately from 4% to 13%)**	The linearity of the detector response was assessed using five standards solutions of Difenoconazole in the concentration range from 0.1027 mg/ml to 0.3081 mg/ml (approximately from 4% to 13%). To prepare the calibration curve volumes of: 0.50 ml, 0.80 ml, 1.00 ml, 1.20 ml and 1.50 ml of standard solution (1.0269 mg/ml) were pipetted to 5 ml flasks and acetonitrile was added up to the mark. y=36019402.7511x-223678.9 R^2 = 0.9997 Correlation coefficient should be $R^2 \geq 0.99$. The obtained result is acceptable.
Precision – Repeatability Mean **n = 6** **(0.22 %RSD)**	The content of difenoconazole in the SNS-F-11 preparation was determined by analysis of six - about 22 mg - portions of the specimen solution. Acceptable relative standard deviation for analyte in preparation (8.157%) is RSDr ≤ 1.96%. The obtained result RSDr = 0.22% and the Horrat value Hr = 0.11 are acceptable.
Accuracy **n = 10** **(100.4% Marginal Recovery)**	Recovery of determination of difenoconazole content in SNS-F-11 preparation was assessed by recovery from ten solutions. To first five 1 ml vials, was added 180 µl (level I) of difenoconazole standard solution at concentration of 1.0848 mg/ml and solution of placebo (concentration ≈ 2,0 mg/ml) was added up to the volume. To each of the remaining five vials, 200 µl (level II) of difenoconazole standard solution (concentration of difenoconazole standard solution – 1.0848mg/ml) were added. Solution of placebo (concentration ≈ 2,0 mg/ml) was added up to the mark. The concentration of analyte in each solution was calculated from the equation of the calibration curve. Level 1: approximately 8% Level 2: approximately 9%

	Difenoconazole
	The result of 100.4% fulfils the acceptance criterion (90 – 110%).
Interference/ Specificity	To prove specificity of the developed method, chromatograms of: solvent, standard, placebo and specimen of SNS-F-11 were performed and superimposed. There are no other peaks that could interfere with peak of diefenoconazole under the specified chromatographic conditions.
Comment	The validation parameters (specificity, linearity, repeatability and recovery) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev.5.

Conclusion

It was confirmed that chromatographic methods of determination of the active substance difenoconazole are specific. No interference was observed. The validation parameters (specificity, linearity, repeatability and recovery) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev.5.

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

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

Reference:	Validation included in the following reports:
Report	Jarosław Kupiec, M.Sc., 2022/ Synthos Agro Validation included in the report: SNS-F-11 Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The content of toluene in the examined preparation was determined using headspace analysis in combination with gas chromatography and flame ionization detection (HS-GC-FID) using external standard-toluene in accordance with the method MT/BA-04/23. The method was developed and validated according to the guide SANCO/3030/99 REV. 5, 22/03/2019.

Reference material

Toluene, 99.94 %, Sigma-Aldrich, batch no. STBH8828

Apparatus and materials

- VARIAN CP-3800 Gas Chromatograph with FID
- Teledyne Tekmar HT-3 Headspace Autosampler

- Rxi®-1301Sil MS capillary column, 30 m × 0.25 mm × 1.0 µm (RESTEK)
- Analytical balance Mettler Toledo XS205 Dual Range, accuracy of 0.01 mg
- Glass pipettes
- Automatic pipettes
- Glass graduated flasks
- 20 mL headspace vials with alumina caps and Teflon-silicone septa
- Laboratory crimper
- Autosampler vials
- Typical laboratory equipment

Reagents

- Dimethyl sulfoxide (DMSO), for headspace analysis, VWR Chemicals

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) SNS-F-11

	Toluene
Author(s), year	Jarosław Kupiec, M.Sc., 2023
Principle of method	The content of toluene in the examined preparation was determined using headspace analysis in combination with gas chromatography and flame ionization detection (HS-GC-FID) using external standard-toluene in accordance with the method MT/BA-04/23. The method was developed and validated according to the guide SANCO/3030/99 REV. 5, 22/03/2019. Chromatographic conditions VARIAN CP-3800 Gas Chromatograph with FID Rxi®-1301Sil MS capillary column, 30 m × 0.25 mm × 1.0 µm (RESTEK) Oven: 45 ºC (1 min), 10 ºC/min → 100 ºC, 30 ºC /min → 200 ºC Carrier gas: Helium Flow: 2 mL/min Inlet temperature: 250 ºC Detector temperature: 300 ºC Split ratio: 1:20 Auxiliary gases flow: nitrogen: 25 mL/min hydrogen: 30 mL/min air: 300 mL/min Headspace Autosampler conditions: Teledyne Tekmar HT-3 Headspace Autosampler Valve oven temperature: 110 ºC Transfer line temperature: 120 ºC Platen/sample temperature: 100 ºC Sample equilibration time: 10 min Mixing time: 3 min (Level 7) Pressurization: 7 psig Loop fill pressure: 5 psig Loop volume: 1 mL Injection time: 1 min Under the above conditions retention time of toluene was 5.65 ± 0.05 min and the total time of analysis is 9.83 min
Linearity	To determine limit of quantification (LOQ) and method linearity stock

	Toluene
Linear between: **0.001 mg/ml to 0.0200 mg/ml** **(approximately from 0.002% to 0.04%)**	solutions of toluene were diluted. DMSO was used as a solvent. Detector response linearity was examined using 6 solutions in the range of toluene content from 0.001 to 0.0200 mg what for 50 mg of SNS-F-11 preparation constitutes from around 5 to 100 % of maximum allowed toluene content in SNS-F-11 preparation (m/m). Because matrix components of the preparation had an effect on partition of toluene between liquid and gaseous phases, the calibration curves were determined using placebo of SNS-F-11 preparation fortified with known amount of toluene standards. y=778609x-13.114 R^2 = 0.9999 Correlation coefficient should be $R^2 \geq 0.99$. The obtained result is acceptable.
Precision – Repeatability Mean **n = 6** **(3.11 %RSD)**	Content of toluene in the specimen was determined by analyzing of six samples of SNS-F-11 preparation. About 50 mg of the specimen were placed in six headspace vials and then 2 mL DMSO was added. Tightly closed vials were analyzed and the content of toluene were calculated using the calibration curve. RSD for substance at the concentration of ~ 0.0051 % should be less than or equal to 5.93%. The obtained result RSDr = 3.11% and the Horrat value Hr = 0.52 are acceptable.
Accuracy **n = 6 (2 levels)** **(99.4% Total Recovery)**	Recovery of the method for toluene determination in SNS-F-11 preparation was assessed by total recovery value at two levels of concentration. • Level I (0.002%)- Six portions about 50 mg of placebo were placed in headspace vials and 0.100 mL of working standard solution C1 and 1.900 mL DMSO was added. • Level II (0.005%)– Six portion about 30 mg of specimen were placed in another six headspace vials and 0.100 mL of working standard solution C1 and 1.900 mL DMSO was added. The result of 99.4 % fulfils acceptance criterion (70 – 130%).
Interference/ Specificity	To prove specificity of the developed method the following headspace chromatograms (HS-GC-FID) were performed and superimposed: DMSO (sample solvent, 2mL), placebo in DMSO (50 mg + 2 ml), specimen (SNS-F-11 in DMSO (50 mg+2 mL)) and standard solution of toluene in DMSO (0.100 mg in 2 mL). There are no other peaks that could interfere with peak of the determined substance under specified chromatographic conditions.
LOQ **as lowest level of recovery**	Limit of quantification (LOQ) of toluene in SNS-F-11 preparation was defined as the lowest quantity of injected standard that gave precise and accurate measurements and is expressed as the lowest toluene amount used for calibration curve. Limit of quantification is 0.0010 mg what corresponds to 0.002 % (0.020 g/kg) of toluene content in SNS-F-11 preparation. %RSD = 3.38 (n=6) Horrat: 0.49 < 1
Comment	The validation parameters (specificity, linearity, LOQ, repeatability and recovery) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev.5.

Conclusion

It was confirmed that chromatographic methods of determination of the relevant impurities (toluene) are specific. No interference was observed. The validation parameters (specificity, linearity, LOQ, repeatability and recovery) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev. 5.

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

With respect to toxicological, eco-toxicological or environmental aspects SNS-F-11 does not contain any relevant formulants. Therefore, a special analytical method and validation is not needed.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are no CIPAC methods available for determination of difenoconazole.

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

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

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**
Winter wheat (Residues)	Primary	0.01 mg/kg	LC-MS/MS	Wójcik M., 2022
	Confirmatory (if required)	LC-MS/MS is known to be highly specific, no confirmatory method is required.		
Oilseed rape (Residues)	Primary	0.01 mg/kg	LC-MS/MS	Schernikau N., 2023 Schernikau N., Kissmann H., 2023
	Confirmatory (if required)	LC-MS/MS is known to be highly specific, no confirmatory method is required.		
Honey (Residues)	Primary	0.01 mg/kg	LC-MS/MS	Schernikau N., 2023
	Confirmatory (if required)	LC-MS/MS is known to be highly specific, no confirmatory method is required.		
Animal products, food of animal origin (Residues)	Primary	0.01 mg/kg (milk) 0.05 mg/kg (muscle, fat, liver, kidney, eggs)	GC-NPD	AG-544A Wurz R.E.M., 1994, DAR of Difenoconazol (May 2006) Annex B.5.2.2
	Confirmatory (if required)	GC-NPD is known to be highly specific, no confirmatory method is required.		

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**
Soil (Ecotoxicology)	Primary	5.0 mg/kg	HPLC-DAD	Wróbel A., 2023
	Confirmatory (if required)	HPLC-DAD is known to be highly specific, no confirmatory method is required.		
Water (Ecotoxicology)	Primary	0.001 mg/L	HPLC-DAD	Hodorek G., 2023 (study codes: W-41-22; W-42-22) Czarnecka M., 2023
	Confirmatory (if required)	HPLC-DAD is known to be highly specific, no confirmatory method is required		
Bee diet (chronic and larval) (Ecotoxicology)	Primary	water: 10 mg/L sucrose solution (bumblebees): 20 mg/L 1% solution of triton in water: 20 mg/L sucrose solution (honeybees): 1 mg/kg	HPLC-DAD	Wojciech A., 2023 (study code B-01-23) Wojciech A., 2023 (study code B-02-23) Wojciech A., 2023 (study code B-03-23) Wojciech A., 2023 (study code B-04-23)
	Confirmatory (if required)	HPLC-DAD is known to be highly specific, no confirmatory method is required		
Terrestrial Plant (Ecotoxicology)	Primary	deionized water: 10 mg/L	HPLC-DAD	Pieczka P., 2024 Gierbuszewska A., 2024
	Confirmatory (if required)	HPLC-DAD is known to be highly specific, no confirmatory method is required		

Component of residue definition: Triazole alanine (TA); 1,2,4-triazole (1,2,4-T); triazole acetic acid (TAA); triazole lactic acid (TLA)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Winter wheat (Residues)	Primary	0.01 mg/kg	LC-MS/MS	Wójcik M., 2022
	Confirmatory (if required)	LC-MS/MS is known to be highly specific, no confirmatory method is required.		
Oilseed rape (Residues)	Primary	0.01 mg/kg	LC-DMS-MS/MS	Schernikau N., 2023 Schernikau N., Kissmann H., 2023
	Confirmatory (if required)	LC-DMS-MS/MS is known to be highly specific, no confirmatory method is required.		
Honey	Primary	0.01 mg/kg	LC-MS/MS	Schernikau N., 2023

Component of residue definition: Triazole alanine (TA); 1,2,4-triazole (1,2,4-T); triazole acetic acid (TAA); triazole lactic acid (TLA)				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
(Residues)	Confirmatory (if required)	LC-MS/MS is known to be highly specific, no confirmatory method is required.		

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substance and relevant impurities in the plant protection product shall be submitted, unless the applicant shows that these methods already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

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

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

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

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Difenoconazole	0.1 mg/kg (wheat)	Regulation (EU) No 2019/552, Annex III Part A
Plant, high acid content		~~Product is not intended for use in these target crops.~~ 0.1 mg/kg	Regulation (EU) No 2019/552
Plant, high protein/high starch content (dry commodities)		0.1 mg/kg (wheat, tricicale)	Regulation (EU) No 2019/552, Annex III Part A
Plant, high oil content		0.05 mg/kg (poppy seed, gold of pleasure seed, sunflower seed) 0.1 mg/kg (soyabean) 0.5 mg/kg (oilseed rape) 0.2 mg/kg (linseed, mustard seed)	Regulation (EU) No 2019/552, Annex III Part A
Plant, difficult matrices		Product is not intended for use in these target crops.	

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
(hops, spices, tea)			
Muscle	Difenoconazole	0.05 mg/kg	Regulation (EU) No 2019/552, Annex III Part A
Milk		0.005 mg/kg	Regulation (EU) No 2019/552, Annex III Part A
Eggs		0.05 mg/kg	Regulation (EU) No 2019/552, Annex III Part A
Fat		0.05 mg/kg (swine, bovine, sheep, goat, equine) 0.1 mg/kg (poultry, other farmed terrestrial animals)	Regulation (EU) No 2019/552, Annex III Part A
Liver, kidney		0.2 mg/kg (swine, bovine, sheep, goat, equine, other farmed terrestrial animals) 0.1 mg/kg (poultry)	Regulation (EU) No 2019/552, Annex III Part A
Soil (Ecotoxicology)	Difenoconazole and CGA 205375 (metabolite) expressed as Difenoconazole	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Difenoconazole	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Difenoconazole	5.6 µg/L	NOEC *Daphnia magna*, EFSA Conclusion, EFSA Journal 2011;9(1):1967
Air	Difenoconazole	48 µg/m^3	AOEL sys: 0.16 mg/kg bw/d; EFSA Conclusion, EFSA Journal 2011;9(1):1967
Tissue (meat or liver)	Difenoconazole	0.01 mg/kg	Default LOQ SANTE/2020/12830, Rev.1
Body fluids		0.01 mg/kg	Default LOQ SANTE/2020/12830, Rev.1

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 difenoconazole in plant matrices is given in the following tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

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

Component of residue definition: 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	Apple, Lettuce 0.01 mg/kg	LC-MS/MS	Steinhauer S., 2004a; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	ILV	Apple, Lettuce 0.01 mg/kg	LC-MS/MS	Schulz H., 2004; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
High acid content	Primary	~~Apple~~ 0.01 mg/kg	LC-MS/MS	Steinhauer S., 2004a; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1 European Standard EN 15662:2008 (CEN, 2008); rev.2018)
	ILV	~~Apple~~ 0.01 mg/kg	LC-MS/MS	Schulz H., 2004; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
High oil content	Primary	**Oilseed rape 0.01 mg/kg**	LC-MS/MS	Steinhauer S., 2004a; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	ILV	**Oilseed rape 0.01 mg/kg**	LC-MS/MS	Schulz H., 2004; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
High protein/high starch content (dry)	Primary	**Wheat (Grain) 0.01 mg/kg**	LC-MS/MS	Steinhauer S., 2004a; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	ILV	**Wheat (Grain) 0.01 mg/kg**	LC-MS/MS	Schulz H., 2004; DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.1
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Difficult (if required, depends on intended use)	Primary	Product is not intended for use in these target crops.		
	ILV			
	Confirmatory (if required)			

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

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 difenoconazole in animal matrices is given in the following tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

Table 5.3-3: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: Difenoconazole ~~and CGA 205375 (metabolite)~~				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk	Primary	~~0.01~~ mg/kg 0.005 mg/kg	LC-MS/MS	Crook S. J., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2 Ryan J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	ILV	~~0.02~~ mg/kg 0.005 mg/kg	GC-NPD	Benazeraf L., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Eggs	Primary	0.01 mg/kg	LC-MS/MS	Crook S. J., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2 Ryan J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	ILV	0.01 mg/kg	GC-NPD	Benazeraf L., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Muscle	Primary	0.01 mg/kg	LC-MS/MS	Crook S. J., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2 Ryan J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	ILV	0.01 mg/kg	GC-NPD	Benazeraf L., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Fat	Primary	0.01 mg/kg	LC-MS/MS	Crook S. J., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2

Component of residue definition: Difenoconazole ~~and CGA 205375 (metabolite)~~				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
				Ryan J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	ILV	0.01 mg/kg	GC-NPD	Benazeraf L., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Kidney, liver	Primary	0.01 mg/kg	LC-MS/MS	Crook S. J., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2 Ryan J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	ILV	0.01 mg/kg	GC-NPD	Benazeraf L., 2004/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.2.2
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary.		

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

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 difenoconazole in soil is given in the following tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

Table 5.3-4: Validated methods for soil (if appropriate)

Component of residue definition: Difenoconazole and CGA 205375 (metabolite) expressed as Difenoconazole			
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/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.1
Confirmatory	The original method is highly specific therefore an additional confirmatory method is not necessary.		

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

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

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

Table 5.3-5: 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	a) 0.05 µg/L b) 0.1 µg/L	a) GC-ECD b) GC-ECD	a) Tribolet R., 1999a/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2 Tribolet R., 1999b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2 b) Tribolet R., 1990/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2
	ILV	No confirmatory method was provided according to DAR of Difenoconazole (May 2006, B.5)		
	Confirmatory	The original method is highly specific therefore an additional confirmatory method is not necessary.		
Surface water	Primary	a) 0.1 µg/L b) 0.1 µg/L	a) GC-ECD b) GC-ECD	a) Tribolet R., 1999a/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2 Tribolet R., 1999b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2 b) Tribolet R., 1990/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2
	Confirmatory	The original method is highly specific therefore an additional confirmatory method is not necessary.		

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

zRMS:
An independent laboratory validation (ILV) of the analytical method for difenoconazole in drinking water is required according to Regulation (EC) No 283/2013 (post registration requirement after renewal of 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 difenoconazole in air is given in the following tables. For the detailed evaluation of new/ additional studies please refer to Appendix 2.

Table 5.3-6: 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	a) 0.99 ng/l b) 1 ng/l	a) LC-MS/MS b) GC-ECD	a) Tummon O.J., 2004b/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.4 b) Tribolet R., 1992/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2 Tribolet R., 1996/ DAR of Difenoconazole; Annex B.5 Analytical methods; Point B.5.3.2
Confirmatory	The original method (a) is highly specific therefore an additional confirmatory method is not necessary.		

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

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

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

Table 5.3-7: Methods for body fluids and tissues (if appropriate)

A method of analysis for body fluids and tissues is not required because the active substance is not classified as toxic or very toxic.

zRMS:
• An analytical method for difenoconazole in body fluids is required according to Regulation (EC) No 283/2013 (post registration requirement after renewal of active substance)

5.3.2.8 Other studies/ information

No other studies or information is provided.

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	Kupiec J.	2023	SNS-F-11 Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storageJarosław Kupiec, M.Sc., 2023 Study code: BF – 13/23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Wójcik M.	2022	Valor 250 EC: Determination of the residues of difenoconazole in winter wheat. Study code: C-08-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) Analytical phase, GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Schernikau N.	2023	Determination of the residues of difenoconazole and triazole derivative metabolites in oilseed rape following one application of Tores 250 EC in four trials in Northern Europe 2023 Study code: S23-103661 Eurofins Agroscience Services Chem GmbH GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Schernikau N., Kissmann H.	2023	Determination of the residues of difenoconazole and triazole derivative metabolites in oilseed rape following one application of SNS-F-11 in four trials in Poland 2023 Study code: S23-103662 Eurofins Agroscience Services Chem GmbH GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Schernikau N.	2023	Determination of the residues of difenoconazole and triazoles derivative metabolites in honey following	N	Synthos Agro

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
			application of Tores 250 EC in four trials in Northern Europe 2023 Study code: SYT-2303 Eurofins Agroscience Services Chem GmbH GLP Unpublished		Sp. z o.o.
KCP 5.1.2	Wróbel A.	2023	SNS-F-11 Earthworm reproduction test (*Eisenia andrei*) Study code: G-09-23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Hodorek G.	2023	SNS-F-11 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test Study code: W-42-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.1.2	Czarnecka M.	2023	SNS-F-11 Rainbow trout, Acute Toxicity Testing Study code: W-45-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wojciech A.	2023	SNS-F-11 Honeybees (*Apis mellifera L.*), Larval Toxicity Test, Repeated Exposure Study code: B-01-23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wojciech A.	2023	SNS-F-11 Bumblebees (*Bombus spp.*), Acute Oral Toxicity Test Study code: B-02-23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP	N	Synthos Agro Sp. z o.o.

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
			Unpublished		
KCP 5.2	Wojciech A.	2023	SNS-F-11 Bumblebees (*Bombus spp.*), Acute Contact Toxicity Test Study code: B-03-23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wojciech A.	2023	SNS-F-11 Honeybees (*Apis mellifera L.*), Chronic Oral Toxicity Test Study code: B-04-23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Hodorek G.	2023	SNS-F-11 *Daphnia magna*, Acute Immobilisation Test Study code: W-41-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Pieczka P.	2024	SNS-F-11 Terrestrial Plant Test: Vegetative Vigour Test Study code: G-46-24 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Gierbuszewska A.	2024	SNS-F-11 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test Study code: G-47-24 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Branch Pszczyna) GLP Unpublished	N	Synthos Agro Sp. z o.o.

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2	Wurz R.E.M.	1994	Analytical method for the determination of CGA 169374 residues in dairy and poultry tissue, eggs and milk by gas chromatography Syngenta Crop Protection AG, Basel, Switzerland Ciba-Geigy Corp., Greensboro, United States, Report No AG-544A Syngenta File N° CGA169374/0933 Not GLP Not Published	N	Syngenta
KCP 5.2	Benazeraf L.	2004	Independent Laboratory Validation of Residue Method REM 147.07 for the Determination of Difenocon-azole and CGA205375 in Animal Products Syngenta Crop Protection AG, Basel, Switzerland ADME - Bioanalyses, Vergèze, France, Report No SYN/DIF/04031 GLP Not Published Syngenta File N° CGA169374/2535	N	Syngenta
KCP 5.2	Crook S. J.	2004	Residue Method for the Determination of Residues of Difenoconazole (CGA169374) and CGA 205375 in Animal Products. Final Determination by LC-MS/MS Syngenta Crop Protection AG, Basel, Switzerland Syngenta, Jealott's Hill, United Kingdom, Report No REM 147.07 Not GLP Not Published Syngenta File N° CGA205375/0021	N	Syngenta
KCP 5.2	Ryan J.	2004b	Difenoconazole (CGA169374) and CGA205375: Validation of Residue Analytical Method REM 147.07 for the Determination of Residues in Animal Products Syngenta Crop Protection AG, Basel, Switzerland Syngenta, Jealott's Hill, United Kingdom, Report No RJ3478B	N	Syngenta

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Not Published Syngenta File N° CGA205375/0020		
KCP 5.2	Schulz H.	2004	Independent Laboratory Validation of DFG Method S19 (Extended Revision) for the Determination of Residues of difenoconazole in/on plant matrices Syngenta Crop Protection AG, Basel, Switzerland Institut Fresenius, Taunusstein, Germany, Report No IF-04/00160619 GLP Not Published Syngenta File N° CGA169374/2507	N	Syngenta
KCP 5.2	Steinhauer S.	2004a	Difenoconazole: Validation of the DFG Method S 19 (Extended Revision) for the Determination of Residues of Difenoconazole in Milk, Meat, Fat, Egg, Liver and Kidney Syngenta Crop Protection AG, Basel, Switzerland Dr. Specht & Partner Chem. Laboratorien GmbH, Hamburg, Germany, Report No SYN-0302V Az. G03-0024 GLP Not Published Syngenta File N° CGA169374/2443	N	Syngenta
KCP 5.2	Tribolet R.	1990	CGA 169374, Determination of residues of parent compound by gas liquid chromatography (GLC), potable water Novartis Crop Protection AG, Switzerland Ciba-Geigy Ltd., Basel, Switzerland Report No REM-147-01 Not GLP, Not Published Syngenta File N° CGA169374/0055	N	Syngenta
KCP 5.2	Tribolet R.	1992	Sampling of air and determination of residues of parent compound by high performance liquid chromatography NCP/Novartis Crop Protection AG, Switzerland Ciba-Geigy Ltd., Basel, Switzerland	N	Syngenta

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
			Report No REM133-03, 15/12/1992 GLP, Not Published Syngenta File N° CGA173506/0234		
KCP 5.2	Tribolet R.	1992	Sampling of air and determination of residues of parent compound by gas chromatography Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No REM-147-02 Not GLP Not Published Syngenta File N° CGA169374/0722	N	Syngenta
KCP 5.2	Tribolet R.	1996	Report on Special Study 102/96. Validation of method REM 147.02 in air, Validation by analytis of fortified specimens and determination of recoveries Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 102/96 GLP Not Published Syngenta File N° CGA169374/1192	N	Syngenta
KCP 5.2	Tribolet R.	1999a	Determination of parent compound by gas chromatography, Water Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No REM 147.05 Not GLP Not Published Syngenta File N° CGA169374/1783	N	Syngenta
KCP 5.2	Tribolet R.	1999b	Validation of method 147.05 by Analysis of Fortified Water Specimens for Difenoconazole (CGA 169374) and Evaluation of Recoveries Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 226/98	N	Syngenta

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
			GLP Not Published Syngenta File N° CGA169374/1782		
KCP 5.2	Tummon O.J.	2004a	Difenoconazole. Validation of an Analytical Method for the Determination of Residues of Difenoconazole and CGA205375 in Soil Syngenta Crop Protection AG, Basel, Switzerland Syngenta, Jealott's Hill, United Kingdom, Report No RJ3459B GLP Not Published Syngenta File N° CGA169374/2501	N	Syngenta
KCP 5.2	Tummon O.J.	2004b	Difenoconazole. Validation of an Analytical Method for the Determination of Residues of Difenoconazole in Air Syngenta Crop Protection AG, Basel, Switzerland Syngenta, Jealott's Hill, United Kingdom, Report No RJ3495B GLP Not Published Syngenta File N° CGA169374/2500	N	Syngenta

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for Difenoconazole

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

No new or additional studies have been submitted.

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

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

~~**A 2.1.2.1.1 Analytical method 1**~~

A 2.1.2.1.1.1 ~~Method validation~~

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Wójcik M., 2022
Report	Analytical phase: Valor 250 EC. Determination of the residues of difenoconazole in winter wheat, Marcin Wójcik, 2022, Study code: C-08-21
Guideline(s):	Yes (SANTE/2020/12830, Rev.1)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of difenoconazole and its metabolites in winter wheat samples was chemically determined with a liquid chromatographic method with mass spectrometer detection. The method for each compound was validated in according to EC Guidance Documents SANTE/2020/12830, rev. 1.

Equipment

- analytical balance, Adventurer Pro AV 114CM
- balance, WPS 510/C
- balance, WTC 200
- laboratory centrifuge, MPW-351e
- volumetric flasks, various volumes

- variable volume single-channel pipettes, various volumes
- glass pipettes
- blender, HGB55E
- liquid chromatograph with mass spectrometer, NEXERA XR LCMS8045
- laboratory deionizer, Elix® Essential 10 UV
- freezer low temperature, ULUF 450
- filter paper middle, 90 mm
- autosampler vials with PTFE/silicone septa and screw caps, clear glass, 2 mL

Analytical procedure - Difenoconazole

Reagents and solvents

- deionized water, Łukasiewicz-IPO
- acetonitrile (LC-MS), J.T. Baker, batch number 2114805856
- formic acid, ≥99% for LC-MS, VWR Chemicals, batch number DB634419
- anhydrous sodium sulphate (VI) pure p.a., J.T Baker, batch number 2106706810
- QuEChERS BEKOlut Citrate-Kit-01, BEKOlut GmbH & Co. KG, batch number 2821
- QuEChERS BEKOlut PSA-Kit-04, BEKOlut GmbH & Co. KG, batch number 4621
- QuEChERS BEKOlut PSA-Kit-08, BEKOlut GmbH & Co. KG, batch number 3621
- 99.8 ± 0.1 % standard of difenoconazole, Łukasiewicz-IPO Warsaw, Poland, batch number 2A/20
- standard solution of difenoconazole at the concentration of 1.0 mg/mL
- working solutions of difenoconazole at the concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 ng/mL

The following chromatographic parameters were used

	Parameter		
Chromatographic System	Shimadzu Nexera XR		
Analytical Column	Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm		
Column Temperature	40°C		
Injection Volume	1 µL		
Mobile Phase A Mobile Phase B	Water / Formic acid (1000:1, v/v) Acetonitrile / Formic acid (1000:1, v/v)		
Flow Rate	0.5 mL/min		
Gradient (including wash and equilibration)	Time [min]	Phase A [%]	Phase B [%]
	0.00 0.50 1.75 2.75 2.80 5.00	90 90 5 5 90 90	10 10 95 95 10 10
Detection System	Shimadzu LCMS-8045 Mass Spectrometer		
Ionisation	Electro Spray (ESI)		
Analyte	**Transitions**		**Polarity**
Difenoconazole	406.10 ->250.95[1)] 406.10 ->337.00[2)]		positive

[1)] Quantitation transition. Mass transition used for quantification.

[2)] Confirmatory transition. The second transition has been monitored but will not reported, except for the validation

experiment.

Sample preparation for the chromatographic analysis

Whole plants

First, 5 g ground (frozen) whole plants of winter wheat was weighed in a 50 mL centrifuge tube, and next 2 mL of deionized water and 10 mL of acetonitrile was added. QuEChERS BEKOlut Cit-rate-Kit-01 was added to the sample mixture and shaken vigorously for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and decanted. QuEChERS BEKOlut PSA-Kit-08 was added to the aliquot and shaken vigorously for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and filtered through anhydrous sodium sulphate (VI). The volume of clear extract was made up to 25 mL with mixture of ace-tonitrile : formic acid (1000:2, v/v). Finally, 1.0 µL of the sample was introduced into a LC-MS column.

Grains

First, 5 g ground (frozen) grains of winter wheat was weighed in a 50 mL centrifuge tube, and next 10 mL of deionized water and 10 mL of acetonitrile was added. QuEChERS BEKOlut Citrate-Kit-01 was added to the sample mixture and shaken vigorously for 0.5 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and decanted. QuEChERS BEKOlut PSA-Kit-04 was added to the aliquot and shaken vigor-ously for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and filtered through anhy-drous sodium sulphate (VI). The volume of clear extract was made up to 25 mL with mixture of acetoni-trile : formic acid (1000:2, v/v). Finally, 1.0 µL of the sample was introduced into a LC-MS column.

Straw

First, 2 g ground (frozen) straw of winter wheat was weighed in a 50 mL centrifuge tube, and next 5 mL of deionized water and 10 mL of acetonitrile was added. QuEChERS BEKOlut Citrate-Kit-01 was added to the sample mixture and shaken vigorously for 1 min. by hand. The sample was cen-trifuge for 5 min. at 3800 rpm and decanted. QuEChERS BEKOlut PSA-Kit-08 was added to the aliquot and shaken vigorous-ly for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and filtered through anhydrous sodium sulphate (VI). The volume of clear extract was made up to 10 mL with mixture of acetonitrile : formic acid (1000:2, v/v). Finally, 1.0 µL of the sample was introduced into a LC-MS column.

Preparation of standard solutions

The stock solution with a concentration of 1.0 mg/mL was prepared by weighting 10.0 mg of stand-ard into a volumetric flask with a capacity of 10 mL, dissolving in acetonitrile for LC-MS, and next the vol-ume was made up to 10 ml with the same solvent. Intermediate standard solutions at concen-tration 10 µg/mL and 0.1 µg/mL were prepared by dilution of stock solution with mixture of ace-tonitrile for LC-MS.

The working solutions were prepared by diluting standards with a higher concentration.

The standard solution was used to prepare working solutions at the concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng/mL.

Analytical procedure – Triazole alanine

Reagents and solvents

- deionized water, Łukasiewicz-IPO
- methanol (LC-MS), POCH, batch number 0818/09/20
- methanol (LC-MS), POCH, batch number 0820/08/20

- formic acid, ≥98%, MERCK, batch number STBH 6570
- formic acid, ≥99% for LC-MS, VWR Chemicals, batch number DB634419
- ethylenediamine tetraacetic acid (EDTA), POCH, batch number 1037/12/21
- active carbon CWZ 22, Elbar-Katowice" Sp. Z o.o., batch number87/10/16

The following chromatographic parameters were used

	Parameter		
Chromatographic System	Shimadzu Nexera XR		
Analytical Column	Arion Polar C18 3µm 100A, l=100 mm, Ø=2.1 mm		
Column Temperature	35°C		
Injection Volume	2 µL		
Mobile Phase A Mobile Phase B	Water / Formic acid (1000:1, v/v) Methanol / Formic acid (1000:1, v/v)		
Flow Rate	0.25 mL/min		
Gradient (including wash and equilibration)	Time [min]	Phase A [%]	Phase B [%]
	0.00 1.25 1.75 2.00 2.05 3.50	90 90 0 0 90 90	10 10 100 100 10 10
Detection System	Shimadzu LCMS-8045 Mass Spectrometer		
Ionisation	Electro Spray (ESI)		
Analyte	**Transitions**		**Polarity**
Triazole alanine	157.00 ->70.15[1)] 157.00 ->88.10[2)]		positive

[1)] Quantitation transition. Mass transition used for quantification.

[2)] Confirmatory transition. The second transition has been monitored but will not reported, except for the validation experiment.

Sample preparation for the chromatographic analysis

Whole plants

First, 5 g ground (frozen) whole plants of winter wheat was weighed in a 50 mL centrifuge tube and 2 mL of water and 10 mL of 1% solution of formic acid in methanol was added. The sample mix-ture was shaken vigorously for 5 min. by hand. Next 100 mg of active carbon was added to the sample and shaken vigorously for 0.5 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter paper. The volume of clean extract was made up to 25 mL with mixture of methanol : formic acid (1000 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Grains

First, 5 g ground (frozen) grains of winter wheat was weighed in a 50 mL centrifuge tube and 9 mL of water was added. The mixture was then allowed to stand for 10 minutes. Next 10 mL of 1% solution of formic acid in methanol and 1 mL of 10% aqueous EDTA solution was added to the sample and shaken vigorously for 10 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter pa-per. The volume of clean extract was made up to 25 mL with mixture of methanol : formic acid (1000 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Straw

First, 2 g ground (frozen) straw of winter wheat was weighed in a 50 mL centrifuge tube and 4 mL of water was added. The mixture was then allowed to stand for 10 minutes. Next 10 mL of 1% solution of formic acid in methanol and 1 mL of 10% aqueous EDTA solution was added to the sample and shaken vigorously for 10 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter pa-per. The volume of clean ex-tract was made up to 10 mL with mixture of methanol : formic acid (1000 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Preparation of standard solutions

The stock solution with a concentration of 1.0 mg/mL was prepared by weighting 10.0 mg of stand-ard into a volumetric flask with a capacity of 10 mL, dissolving in deionized water, and next the volume was made up to 10 ml with the same solvent. Intermediate standard solutions at concentra-tion 10 µg/mL, 1 µg/mL and 0.1 µg/mL were prepared by dilution of stock solution with deionized water.
The working solutions were prepared by diluting standards with a higher concentration.
The standard solution was used to prepare working solutions at the concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng/mL.

Analytical procedure – 1,2,4-triazole, triazole acetic acid, triazole lactic acid

Reagents and solvents

- deionized water, Łukasiewicz-IPO
- acetonitrile (LC-MS), J.T. Baker, batch number 2109605850
- acetonitrile (LC-MS), J.T. Baker, batch number 2114805856
- formic acid, ≥98%, MERCK, batch number STBH 6570
- formic acid, ≥99% for LC-MS, VWR Chemicals, batch number DB634419
- ethylenediamine tetraacetic acid (EDTA), POCH, batch number 1037/12/21
- active carbon CWZ 22, Elbar-Katowice" Sp. Z o.o., batch number87/10/16

The following chromatographic parameters were used

	Parameter		
Chromatographic System	Shimadzu Nexera XR		
Analytical Column	Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm		
Column Temperature	30°C		
Injection Volume	2 µL		
Mobile Phase A Mobile Phase B	Water / Formic acid (1000:1, v/v) Acetonitrile / Formic acid (1000:1, v/v)		
Flow Rate	0.4 mL/min		
Gradient (including wash and equilibration)	Time [min]	Phase A [%]	Phase B [%]
	0.00 0.75 1.50 1.75 1.80 3.00	90 90 5 5 90 90	10 10 95 95 10 10
Detection System	Shimadzu LCMS-8045 Mass Spectrometer		
Ionisation	Electro Spray (ESI)		

Analyte	**Transitions**	**Polarity**
1,2,4-triazole	70.20 > 70.10[1)] 70.20 > 43.20[2)]	positive
triazole acetic acid	128.20 > 70.20[1)] 128.20 > 43.10[2)]	positive
triazole lactic acid	158.20 > 70.10[1)] 158.20 > 42.95[2)]	positive

[1)] Quantitation transition. Mass transition used for quantification.
[2)] Confirmatory transition. The second transition has been monitored but will not reported, except for the validation experiment.

Sample preparation for the chromatographic analysis

Whole plants

First, 5 g ground (frozen) whole plants of winter wheat was weighed in a 50 mL centrifuge tube and 2 mL of water and 10 mL of 1% solution of formic acid in acetonitrile was added. The sample mix-ture was shaken vigorously for 5 min. by hand. Next 100 mg of active carbon was added to the sample and shaken vigorously for 0.5 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter paper. The volume of clean ex-tract was made up to 20 mL with mixture of acetonitrile : deionized water : formic acid (500 : 500 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Grains

First, 5 g ground (frozen) grains of winter wheat was weighed in a 50 mL centrifuge tube and 9 mL of water was added. The mixture was then allowed to stand for 10 minutes. Next 10 mL of 1% solution of formic acid in acetonitrile and 1 mL of 10% aqueous EDTA solution was added to the sample and shaken vigorously for 10 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter pa-per. The volume of clean ex-tract was made up to 20 mL with mixture of acetonitrile : deionized water : formic acid (500 : 500 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Straw

First, 2.5 g ground (frozen) straw of winter wheat was weighed in a 50 mL centrifuge tube and 4 mL of water was added. The mixture was then allowed to stand for 10 minutes. Next 10 mL of 1% solution of formic acid in methanol and 1 mL of 10% aqueous EDTA solution was added to the sample and shaken vigorously for 10 min. by hand. The sample was place into a freezer for 30 min at ca. -80°C. Still cold sample was centrifuge for 5 min. at 3800 rpm and filtered through filter pa-per. The volume of clean ex-tract was made up to 10 mL with mixture of acetonitrile : deionized water : formic acid (500 : 500 : 1, v/v). Finally, 2.0 µL of the sample was introduced into a LC-MS column.

Preparation of standard solutions

stock solution with a concentration of 1.0 mg/mL was prepared for 1,2,4-triazole, triazole acetic acid, triazole lactic acid individually by weighting 10.0 mg of standards into a volumetric flask with a capacity of 10 mL, dissolving in deionized water, and next the volume was made up to 10 ml with the same sol-vent. Intermediate standard solutions of standards mixture at concentration 10 µg/mL 1.0 µg/mL and 0.1 µg/mL were prepared by dilution of stock solutions with acetonitrile.
The working solutions were prepared by diluting standards with a higher concentration.
The standard solution was used to prepare working solutions at the concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100 ng/mL.

Results and discussions

Table A 1: Recovery results from method validation of difenoconazole and triazole metabolites using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%) Quantitation transition	RSD (%) Quantitation transition	Mean recovery (%) Confirmatory transition	RSD (%) Confirmatory transition	Comments
Wheat whole plants	Difenoconazole	0.01 mg/kg (n=5)	84.0%	2.3%	77.8%	6.9%	The mean recovery at each fortification level should be in the range of 70 – 120%. The relative standard deviation should be ≤ 20% for each level
		0.1 mg/kg (n=5)	94.5%	1.3%	94.1%	1.7%	
	Triazole alanine	0.01 mg/kg (n=5)	98.6%	1.9%	97.3%	2.7%	
		0.1 mg/kg (n=5)	95.8%	1.7%	95.7%	2.9%	
	1,2,4-triazole	0.01 mg/kg (n=5)	79.4%	4.2%	84.0%	3.1%	
		0.1 mg/kg (n=5)	96.2%	2.1%	95.0%	2.7%	
	Triazole acetic acid	0.01 mg/kg (n=5)	83.2%	8.2%	81.0%	6.2%	
		0.1 mg/kg (n=5)	94.3%	2.4%	90.0%	2.1%	
	Triazole lactic acid	0.01 mg/kg (n=5)	81.7%	4.0%	74.8%	3.5%	
		0.1 mg/kg (n=5)	93.6%	1.8%	94.3%	3.0%	
Wheat grains	Difenoconazole	0.01 mg/kg (n=5)	78.6%	3.1%	80.7%	5.3%	
		0.1 mg/kg (n=5)	90.6%	1.9%	89.4%	3.2%	
	Triazole alanine	0.01 mg/kg (n=5)	97.3%	4.7%	97.5%	4.6%	
		0.1 mg/kg (n=5)	94.2%	3.3%	95.4%	2.5%	
	1,2,4-triazole	0.01 mg/kg (n=5)	78.0%	6.9%	74.7%	2.1%	

Matrix	Analyte	Fortification level (mg/kg) (*n* = x)	Mean recovery (%) Quantitation transition	RSD (%) Quantitation transition	Mean recovery (%) Confirmatory transition	RSD (%) Confirmatory transition	Comments
		0.1 mg/kg (n=5)	95.0%	1.3%	88.4%	4.1%	
	Triazole acetic acid	0.01 mg/kg (n=5)	78.0%	6.9%	76.0%	8.2%	
		0.1 mg/kg (n=5)	95.0%	1.3%	94.3%	2.9%	
	Triazole lactic acid	0.01 mg/kg (n=5)	79.9%	5.3%	80.0%	10.4%	
		0.1 mg/kg (n=5)	93.8%	2.1%	92.8%	2.8%	
Wheat straw	Difenoconaz-ole	0.01 mg/kg (n=5)	82.9%	3.0%	78.4%	5.7%	
		0.1 mg/kg (n=5)	92.8%	1.1%	92.4%	2.3%	
	Triazole alanine	0.01 mg/kg (n=5)	88.8%	3.5%	86.8%	5.2%	
		0.1 mg/kg (n=5)	92.5%	3.7%	92.6%	3.9%	
	1,2,4-triazole	0.01 mg/kg (n=5)	76.4%	4.8%	84.5%	5.0%	
		0.1 mg/kg (n=5)	92.5%	1.9%	91.9%	2.9%	
	Triazole acetic acid	0.01 mg/kg (n=5)	84.7%	4.0%	81.2%	8.9%	
		0.1 mg/kg (n=5)	92.4%	1.4%	88.4%	4.2%	
	Triazole lactic acid	0.01 mg/kg (n=5)	82.4%	5.1%	87.1%	14.4%	
		0.1 mg/kg (n=5)	92.4%	1.2%	96.4%	2.3%	

Table A 2: Characteristics for the analytical method used for validation of difenoconazole and triazole metabolites residues in whole plant of winter wheat

	Difenoconazole	**TA**	**1,2,4-T**	**TAA**	**TLA**
Specificity	It was found out that no signals of difenoconazole and TA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.0025 mg/kg.		It was found out that no signals of 1,2,4-T, TAA, TLA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.004 mg/kg		
Calibration (type, number of data points)	5 levels of calibration				
Calibration range	0.5 ng/mL – 50 ng/mL	0.5 ng/mL – 50 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL
Assessment of matrix effects is presented	Yes Matrix effect on the detection of difenoconazole in extracts of whole plant of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -0.03 and 2.2 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of whole plant of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -16.7 and -15.9 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of whole plant of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -4.3 and -4.2 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of whole plant of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -6.5 and -4.0 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of whole plant of winter wheat was found to be insignificant (≤ 20%). Matrix effect = 7.1 and 15.7% (for standard 2.5 ng/mL).
Limit of determination/quantification	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg

Table A 3: Characteristics for the analytical method used for validation of difenoconazole and triazole metabolites residues in grains of winter wheat

	Difenoconazole	**TA**	**1,2,4-T**	**TAA**	**TLA**
Specificity	It was found out that no signals of difenoconazole and TA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.0025 mg/kg.		It was found out that no signals of 1,2,4-T, TAA, TLA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.004 mg/kg		
Calibration (type, number of data points)	5 levels of calibration				
Calibration range	0.5 ng/mL – 50 ng/mL	0.5 ng/mL – 50 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL

	Difenoconazole	TA	1,2,4-T	TAA	TLA
Assessment of matrix effects is presented	Yes Matrix effect on the detection of difenoconazole in extracts of grains of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -0.85 and 3.6 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of grains of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -17.5 and -18.6 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of grains of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -12.4 and -10.8 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of grains of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -7.5 and -11.6 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of grains of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -3.8 and 6.4 % (for standard 2.5 ng/mL).
Limit of determination/quantification	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg

Table A 4: Characteristics for the analytical method used for validation of difenoconazole and triazole metabolites residues in straw of winter wheat

	Difenoconazole	TA	1,2,4-T	TAA	TLA
Specificity	It was found out that no signals of difenoconazole and TA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.0025 mg/kg.		It was found out that no signals of 1,2,4-T, TAA, TLA were overlapping with the matrix signals of the control samples under the experimental condition, LOD = 0.004 mg/kg		
Calibration (type, number of data points)	5 levels of calibration				
Calibration range	0.5 ng/mL – 50 ng/mL	0.5 ng/mL – 50 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL	1.0 – 100.0 ng/mL
Assessment of matrix effects is presented	Yes Matrix effect on the detection of difenoconazole in extracts of straw of winter wheat was found to be insignificant (≤ 20%). Matrix effect = 7.7 and 12.4 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of straw of winter wheat was found to be insignificant (≤ 20%). Matrix effect = -15.3 and -16.6 % (for standard 2.0 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of straw of winter wheat was found to be insignificant (≤ 20%). Matrix effect = 0.5 and -3.9 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of straw of winter wheat was found to be insignificant (≤ 20%). Matrix effect = 4.6 and 5.0 % (for standard 2.5 ng/mL).	Yes Matrix effect on the detection of difenoconazole in extracts of straw of winter wheat was found to be insignificant (≤ 20%). Matrix effect = 8.0 and 13.1 % (for standard 2.5 ng/mL).
Limit of determination/quantification	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg

Conclusion

Validation parameters were determined in relation to the requirements SANTE/2020/12830, rev.1. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2

A 2.1.2.1.2 Analytical method 2

A 2.1.2.1.2.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Nina Schernikau, 2023 Nina Schernikau, Harry Kissmann, 2023
Report	Determination of the residues of difenoconazole and triazole derivative metabolites in oilseed rape following one application of Tores 250 EC in four trials in Northern Europe 2023, Nina Schernikau, 2023, Study code: S23-103661 Determination of the residues of difenoconazole and triazole derivative metabolites in oilseed rape following one application of SNS-F-11 in four trials in Poland 2023, Nina Schernikau, Harry Kissmann, 2023, Study code: S23-103662
Guideline(s):	SANTE/2020/12830, rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for **difenoconazole** is based on the multi-residue method QuEChERS. In brief, for the analysis of difenoconazole samples of oilseed rape were extracted with acetonitrile after addition of water. The ratio was 2 mL of extraction solvent per g of matrix. Clean-up of the extract was performed by dispersive SPE with primary/secondary amine (PSA) and freezing out the fat for seed. The sample concentration in final extract was 0.025 g sample per mL of extract. Quantification was performed by use of LC-MS/MS detection.

The analytical method for **triazole derivative metabolites** is based on the BCS method 01062/M004 procedure. In brief, for triazole derivative metabolites (TDMs; 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA)) samples of oilseed rape were extracted with methanol/water (4+1, v+v). The ratio was 20 mL of extraction solvent per g of matrix. An aliquot was transferred to a new tube, isotopically labelled internal standard(s) were added prior to evaporating the methanol phase of the extract. Clean-up was carried out by dispersive SPE with C18 material and Celite. The sample concentration in final extract was 0.1 g sample per mL of extract. Quantification was performed by use of liquid chromatography coupled to differential mobility spectrometry – tandem mass spectrometry (LC-DMS-MS/MS) detection with isotopically labelled internal standard(s).

Analytical Method for Difenoconazole

Reagents and materials

- Acetonitrile gradient grade
  Acetonitrile 99.9%, VWR, Art. No. 20060.320
- Formic Acid LC-MS 1, Supelco, Art. No. 533002.0050
- di-Sodium hydrogen citrate 1,5-hydrate 99 -104%, Sigma-Aldrich, Art. No. 1.12264.0500
- Magnesium sulphate ≥98%, VWR, Art. No. 291186R
- PSA polymerically bonded, ethylenediamine-N-propyl phase with primary and secondary amines (Bondesil PSA 40µm, Agilent, Art. No. 12213025)
- Sodium chloride 99.5-100.5%, VWR, Art. No. 27810.295
- tri-Sodium citrate dihydrate 99.0-101.0%, VWR, Art. No. 27833.294
- Water, HiPerSolv CHROMANORM® for HPLC, VWR, Art. No. 23595.328

Preparation of standard solutions

Stock solution(s) of the analyte(s) were prepared by dissolving a weight of the reference item(s) with the aid of an ultrasonic bath. Each stock solution was allocated a unique reference number.
Solutions for fortification and calibration were obtained by (serial) dilution of the stock solution(s).
Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions.
All solutions were stored at typically 1 °C to 10 °C in a glass vial in the dark.

Sample Weight(s) and Fortifications

Control (untreated) samples were fortified prior to extraction with the fortification solutions as described below. The solvent was allowed to evaporate before starting the extraction procedure.

- Study code: S23-103661

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level (mg/kg)
Difenoconazole	Oilseed rape (whole plant)	5.0 ± 0.05	Z02	500	0.100	0.010
		5.0 ± 0.05	Z01	5,000	0.100	0.10
		5.0 ± 0.05	SL17766	400,000	0.050	4.0
	Oilseed rape (seed)	5.0 ± 0.05	Z02	500	0.100	0.010
		5.0 ± 0.05	Z01	5,000	0.100	0.10

- Study code: S23-103662

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level (mg/kg)
Difenoconazole	Oilseed rape (whole plant)	5.0 ± 0.05	Z02	500	0.100	0.010
		5.0 ± 0.05	Z01	5,000	0.100	0.10
		5.0 ± 0.05	SL17766	400,000	0.025	2.0
	Oilseed rape	5.0 ± 0.05	Z02	500	0.100	0.010

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level (mg/kg)
	(seed)	5.0 ± 0.05	Z01	5,000	0.100	0.10

Analytical method for Triazole Metabolites

Reagents and materials

- Bakerbond Octadecyl (C18) 40µm Prep LC, J.T. (BAKER, Art. No. 7025-00)
- Celite 545, Diatomaceous earth (VWR, Art. No. SERA16391.04)
- Formic acid 98.0% (Merck, Art. No. 1002642500)
- Methanol 99.9% (Merck, Art. No. 1.0618.2500)
- Water, distilled

Preparation of standard solutions

Stock solution(s) of the analytes and isotopically labelled standards were prepared by dissolving a weight of the reference items with the aid of an ultrasonic bath. Each stock solution was allocated a unique reference number.
Solutions for fortification and calibration were obtained by (serial) dilution of the stock solution(s).
All solutions were stored at typically 1 °C to 10 °C in a glass vial in the dark

Sample Weight(s) and Fortifications

Control (untreated) samples were fortified prior to extraction with the fortification solutions as described below.
The analytes were fortified jointly.

- Study code: S23-103661

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of mixed fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level (each) (mg/kg)
1,2,4-Triazole, Triazole Alanine, Triazole Lactic Acid Triazole Acetic Acid	Oilseed rape (whole plant)	5.0 ± 0.05	Z112, Z132	100 (each)	0.50	0.010
		5.0 ± 0.05	Z111, Z131	1,000 (each)	0.50	0.10
Triazole Alanine		5.0 ± 0.05	Z131	1,000	1.0	0.20
1,2,4-Triazole, Triazole Alanine, Triazole Lactic Acid Triazole Acetic Acid	Oilseed rape (seed)	5.0 ± 0.05	Z112, Z122, Z132	100 (each)	0.50	0.010
		5.0 ± 0.05	Z111, Z121, Z131	1000 (each)	0.50	0.10
Triazole Alanine		5.0 ± 0.05	SL17792	400,000	0.050	4.0

- Study code: S23-103662

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of mixed fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level (each) (mg/kg)
1,2,4-Triazole, Triazole Alanine, Triazole Lactic Acid Triazole Acetic Acid	Oilseed rape (whole plant)	5.0 ± 0.05	Z132	100 (each)	0.50	0.010
		5.0 ± 0.05	Z131	1000 (each)	0.50	0.10
		5.0 ± 0.05	Z131	1000 (each)	1.00	0.20
Triazole Metabolites	Oilseed rape (seed)	5.0 ± 0.05	Z132	100	0.50	0.01
		5.0 ± 0.05	Z131	1000	0.50	0.10
Triazole Alanine		5.0 ± 0.05	SL 17792	400 000	0.025	2.0

Results and discussions

Table A 5: Recovery results from method validation of difenoconazole and triazole metabolites using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
oilseed rape (whole plant)	Difenoconazole m/z 406→251*	0.01	97	2.5	-
		0.1	90	1.1	-
	Difenoconazole m/z 406→188	0.01	98	3.8	-
		0.1	94	1.4	-
	1,2,4-Triazole m/z 70→43 (Hypercarb)*	0.01	99	7.7	-
		0.1	97	2.8	-
	1,2,4-Triazole m/z 70→43 (Aquasil)	0.01	92	13	-
		0.1	90	7.1	-
	Triazole alanine m/z 157→70 (Hypercarb)*	0.01	85	6.0	-
		0.1	89	1.6	-
	Triazole alanine m/z 157→88 (Hypercarb)	0.01	85	4.2	-
		0.1	84	3.1	-
	Triazole lactic acid m/z 158→70 (Hypercarb)*	0.01	90	6.8	-
		0.1	96	2.5	-
	Triazole lactic acid m/z 158→70 (Aquasil)	0.01	89	13	-
		0.1	98	2.9	-
	Triazole acetic acid	0.01	91	4.6	-

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
	m/z 128→70 (Hypercarb)*	0.1	95	3.0	-
	Triazole acetic acid m/z 128→70 (Aquasil)	0.01	87	14	-
		0.1	100	4.2	-
oilseed rape (seed)	Difenoconazole m/z 406→251*	0.01	77	1.8	-
		0.1	75	3.6	-
	Difenoconazole m/z 406→188	0.01	79	1.7	-
		0.1	74	3.1	-
	1,2,4-Triazole m/z 70→43 (Hypercarb)*	0.01	92	6.1	-
		0.1	85	2.7	-
	1,2,4-Triazole m/z 70→43 (Aquasil)	0.01	86	8.9	-
		0.1	77	4.3	-
	Triazole alanine m/z 157→70 (Hypercarb)*	0.01	76	26	-
		0.1	79	5.1	-
	Triazole alanine m/z 157→88 (Hypercarb)	0.01	79	19	-
		0.1	82	6.3	-
	Triazole lactic acid m/z 158→70 (Hypercarb)*	0.01	81	4.5	-
		0.1	94	2.6	-
	Triazole lactic acid m/z 158→70 (Aquasil)	0.01	90	2.3	-
		0.1	95	6.1	-
	Triazole acetic acid m/z 128→70 (Hypercarb)*	0.01	80	4.0	-
		0.1	95	1.6	-
	Triazole acetic acid m/z 128→70 (Aquasil)	0.01	80	6.2	-
		0.1	95	3.8	-

* Proposed to be used for quantification

Table A 6: Characteristics for the analytical method used for validation of difenoconazole and triazole metabolites residues in oilseed rape

	Difenoconazole	Triazole metabolites
Specificity	The analyte difenoconazole was determined in the final sample extracts by use of LC MS/MS detection with evaluation of one mass transition. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of target analyte. Untreated samples for accompanying control	For triazole alanine, one mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity for validation samples but was not used for quantification of samples. For 1,2,4-triazole, triazole acetic acid and triazole lactic acid, one mass transition was evaluated. A confirmatory run was performed on a

	Difenoconazole	Triazole metabolites
	sample work up, for determination of (concurrent) recoveries and, if needed, for preparation of matrix-matched calibration standards were obtained from the current study or from other GLP studies. The blank values at the expected retention time of difenoconazole resulting from reagents and/or the control sample material used for recovery determinations and for preparation of matrix-matched calibration standards did not exceed a level that would correspond to 30 % of the LOQ. Correction for blank values was not performed.	different stationary phase for validation samples but was not used for quantification of samples. For each of the internal standards of 1,2,4-triazole, TA, TAA and TLA one mass transition was evaluated. Untreated samples for accompanying control sample work up and for determination of (concurrent) recoveries were obtained from other GLP studies. No residues above 30 % of the LOQ were detected for 1,2,4-T and TAA in the control (untreated) samples of oilseed rape (whole plant and seed) used for recovery determinations. However, for the determination of residues of TDMs the recoveries were corrected by subtraction of control sample area from the respective recovery area.
Calibration (type, number of data points)	Minimum 5 levels of calibration.	
Calibration range	0.075 ng/mL to 7.5 ng/mL	0.30 ng/mL to 30 ng/mL
Assessment of matrix effects is presented	The effect of oilseed rape (whole plant and seed) matrix on the detector response of difenoconazole was assessed by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at the same nominal concentrations. Matrix suppression or enhancement was < 20 % for oilseed rape (whole plant) and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the analytical phase (except for sample extracts that were diluted with final solvent to be within the calibration range). Matrix effects were ≥ ± 20 % and deemed to be significant for oilseed rape (seed). Therefore, matrix-matched standards were used for quantification throughout the analytical phase.	Isotopically labelled internal standard was used for quantification of the triazole metabolites 1,2,4-triazole, TA, TAA and TLA so that possible matrix effects on the detector response are automatically compensated when using the response ratio of analyte to internal standard for quantification. Therefore, matrix effects on detection were not determined within this analytical phase.
Limit of determination/ quantification	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg

Conclusion

The methods were successfully validated for determination of all analytes in oilseed rape with an LOQ of 0.01 mg/kg and up to 0.1 mg/kg according to guidance document(s) SANTE/2020/12830, rev. 2.

With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the samples of the study.

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

No new or additional studies have been submitted.

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

A 2.1.2.3.1 Analytical method 1

A 2.1.2.3.1.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following reports: Anna Wróbel, 2023
Report	SNS-F-11 Earthworm reproduction test (*Eisenia andrei*), Anna Wróbel, 2022, Study code: G-09-23
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determinations were accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 1. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2.

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration with mixture of acetonitrile for HPLC and deionized water (50:50, v/v) (D1). The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]

1000 (Stock)	0.1	1	100.0[1)]
1000 (Stock)	0.05	1	50.0[1)]
100.0	0.1	1	10.0[1)]
50.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]

1) Concentration level used for calibration. The range of linearity 1.0 mg difenoconazole/L to 100.0 mg difenoconazole/L.
D1 - mixture of acetonitrile for HPLC and deionized water (50:50, v/v)

Preparation of Fortified Sample

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [g]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	artificial soil	2	10.0	-	-	0.0
Fortification (LOQ)		5	10.0	100	0.5	5.0
Fortification (10x LOQ)		5	10.0	1000 (difenoconazole stock solution)	0.5	50.0

Sample preparation for the chromatographic analysis

First, 5 mL of mixture of acetonitrile for HPLC and deionized water (50:50, v/v), was added to 10 g of artificial soil sample and shaken for 5 minutes and sonicated for 10 minutes. The sample was centrifuged and filtered through filter paper. Then extraction was repeated with 5 mL of mixture of acetonitrile for HPLC and deionized water (50:50, v/v). Finally, an aliquot of the mixed extract was transferred into a HPLC vial for further quantification using HPLC-DAD. The sample was diluted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v) (if necessary).

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev. 2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Ortho-phosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
		Honeywell Fluka	M2520	28.08.2024
Acetonitrile	HPLC	POCH	0314/09/22	09.2025
		POCH	0318/08/22	08.2025
		VWR Chemicals	22L294003	27.12.2025
		Chempur	230606126	06.2025

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 1, 5, 10, 50 and 100 µg/mL in mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Balance	WPS 510/C	Radwag (Poland)
Balance	PS600.X2	Radwag (Poland)
Ultrasonic bath	Sonic-5	Polsonic (Poland)
Laboratory timer	-	TFA Dostmann GmbH & Co. KG
Laboratory centrifuge	MPW-351e	MPW Med. Instruments
Qualitative filter paper	Fi [mm], 90	Chemland (Poland)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 5µm C18 100Å, l = 150 mm, □ = 4,6 mm
Oven temperature	35°C
Injection Volume	10 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
artificial soil	difenoconazole	5.0	86.5	1.4	-
		50.0	88.3	1.8	-

Table A 8: Characteristics for the analytical method used for validation of difenoconazole residues in artificial soil

<table>
<tr><th></th><th>Difenoconazole</th></tr>
<tr><td>Specificity</td><td>The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix control sample.</td></tr>
<tr><td>Calibration (type, number of data points)</td><td>Working solutions of difenoconazole at the concentrations 1.0, 5.0, 10.0, 50.0 and 100.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</td></tr>
<tr><td>Calibration range</td><td>The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.
<table>
<tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr>
<tr><td>difenoconazole</td><td>5979.62</td><td>-143.317</td><td>0.9992678</td></tr>
</table></td></tr>
<tr><td>Assessment of matrix effects is presented</td><td>Yes
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.</td></tr>
<tr><td>Limit of determination/quantification</td><td>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:
<table>
<tr><th>Analyte</th><th>LOQ [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th><th>LOD [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th></tr>
<tr><td>difenoconazole</td><td>5.0</td><td>5.0</td><td>1.0</td><td>1.0</td></tr>
</table></td></tr>
</table>

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830 rev. 1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2

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

A 2.1.2.4.1 Analytical method 1

A 2.1.2.4.1.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following reports: Grażyna Hodorek, 2023
Report	SNS-F-11 *Daphnia magna*, Acute Immobilisation Test, Grażyna Hodorek, 2023, Study code: W-41-22
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 1. Validated method is also compatible to requirements of SANTE/2020/12830 rev. 2

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration. The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0
100.0	0.05	1	10.0
100.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]
5.0	0.1	1	0.5[1)]
1.0	0.1	1	0.1[1)]
0.5	0.1	1	0.05[1)]

1) Concentration level used for calibration. The range of linearity 0.05 mg difenoconazole/L to 5.0 mg difenoconazole/L

D1 - mixture of acetonitrile for HPLC and deionized water (50:50, v/v)

Preparation of Fortified Sample

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	Elendt m7 medium	2	100.0	-	-	0.000
Fortification (LOQ)		5	100.0	100	0.01	0.001
Fortification (10x LOQ)		5	100.0	1000	0.01	0.01

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was acidified with 4 drops of hydrochloric acid to pH ≤ 2, and next sample was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of methanol pure p.a., twice with 5 mL of deionized water, pH ≤ 2 (acidified by hydrochloric acid). Following the sample introduction the column was dried for 5 minutes by vacuum. The part of sample with affinity to the column was eluted twice with 5 mL of methanol pure p.a. and with 5 mL of acetonitrile pure p.a.. Eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in mixture of acetonitrile for HPLC and deionized water (50:50; v/v) applied to chromatographic column

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev.2.

Conditions of the chemical determinations

Chemicals:

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Ortho-phosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025
Acetonitrile	Pure p.a.	POCH	0302/07/21	07.2026
Methanol	Pure p.a.	POL-AURA	CTH657	16.05.2025
Hydrochloric acid 37%	ACS reagent	Sigma-Aldrich	STBK 7572	04.2024
SPE column Supelclean ENVI-18	-	Supelco	15832401	12.01.2028

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- deionized water, pH ≤ 2 (acidified by hydrochloric acid),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v),
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 100, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL in mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Measuring cylinder	Various volumes	Different supliers
Rotary vacuum evaporator with water medium bath	RV 05 basic HB 4 basic	IKA - WERKE
Rotary vacuum evaporator with water medium bath	RV 10 digital HB 10 digital	IKA - WERKE
SPE manifold	-	Supelco
Adapter for SPE	-	Baker
Laboratory timer	-	TFA Dostmann GmbH & Co. KG
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 5µm C18 100Å, l = 150 mm, □ = 4,6 mm
Oven temperature	35°C
Injection Volume	20 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 9: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Elendt m7 medium	difenoconazole	0.001	108.4	1.4	-
		0.01	104.3	2.6	-

Table A 10: Characteristics for the analytical method used for validation of difenoconazole residues in Elendt m7 medium

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and

<table>
<tr><th></th><th>Difenoconazole</th></tr>
<tr><td></td><td>would be observed in the chromatograms of the matrix control sample.</td></tr>
<tr><td>Calibration (type, number of data points)</td><td>Working solutions of difenoconazole at the concentrations 0.05, 0.1, 0.5, 1.0, 5.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</td></tr>
<tr><td>Calibration range</td><td>The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.
<table>
<tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr>
<tr><td>difenoconazole</td><td>126401</td><td>119.876</td><td>0.9999698</td></tr>
</table></td></tr>
<tr><td>Assessment of matrix effects is presented</td><td>Yes
Matrix effect was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.</td></tr>
<tr><td>Limit of determination/quantification</td><td>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:
<table>
<tr><th>Analyte</th><th>LOQ [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th><th>LOD [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th></tr>
<tr><td>difenoconazole</td><td>0.001</td><td>0.1</td><td>0.0005</td><td>0.05</td></tr>
</table></td></tr>
</table>

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830 rev.1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2

A 2.1.2.4.2 Analytical method 2

A 2.1.2.4.2.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference: Validation included in the following reports:
Grażyna Hodorek, 2023
Małgorzata Czarnecka, 2023

Report: SNS-F-11 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test, Grażyna Hodorek, 2023, Study

	code: W-42-22 SNS-F-11 Rainbow trout, Acute Toxicity Testing, Małgorzata Czarnecka, 2023, Study code: W-45-22
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 1. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2.

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration. The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0
100.0	0.05	1	10.0
100.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]
5.0	0.1	1	0.5[1)]
1.0	0.1	1	0.1[1)]
0.5	0.1	1	0.05[1)]

1) Concentration level used for calibration. The range of linearity 0.05 mg difenoconazole/L to 5.0 mg difenoconazole/L

D1 - mixture of acetonitrile for HPLC and deionized water (50:50, v/v)

Preparation of Fortified Sample

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	water	2	100.0	-	-	0.000
Fortification		5	100.0	10	0.01	0.001

(LOQ)						
Fortification (10x LOQ)		5	100.0	100	0.01	0.01

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was acidified with 4 drops of hydrochloric acid to pH ≤ 2, and next sample was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of methanol pure p.a., twice with 5 mL of deionized water, pH ≤ 2 (acidified by hydrochloric acid). Following the sample introduction the column was dried for 5 minutes by vacuum. The part of sample with affinity to the column was eluted twice with 5 mL of methanol pure p.a. and with 5 mL of acetonitrile pure p.a.. Eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in mixture of acetonitrile for HPLC and deionized water (50:50; v/v) applied to chromatographic column.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev. 2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Ortho-phosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025
		VMR Chemicals	22L294003	27.12.2025
Acetonitrile	Pure p.a.	POCH	0302/07/21	07.2026
Methanol	Pure p.a.	POL-AURA	CTH657	16.05.2025
Hydrochloric acid 37%	ACS reagent	Sigma-Aldrich	STBK 7572	04.2024
		WARCHEM	05346/12/22	12.2026
SPE column Supelclean ENVI-18	-	Supelco	15832401	12.01.2028
			15905201	05.06.2028
			16237201	17.07.2028

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- deionized water, pH ≤ 2 (acidified by hydrochloric acid),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v),
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 100, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL in mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Equipment

Equipment	Size,	Manufacturer/Supplier

	Description	
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Measuring cylinder	Various volumes	Different supliers
Rotary vacuum evaporator with water medium bath	RV 05 basic HB 4 basic	IKA - WERKE
Rotary vacuum evaporator with water medium bath	RV 10 digital HB 10 digital	IKA - WERKE
SPE manifold	-	Supelco
Adapter for SPE	-	Baker
Laboratory timer	-	TFA Dostmann GmbH & Co. KG
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 5µm C18 100Å, l = 150 mm, □ = 4,6 mm
Oven temperature	35°C
Injection Volume	20 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
water	difenoconazole	0.001	93.4	2.4	-
		0.01	94.8	2.1	-

Table A 12: Characteristics for the analytical method used for validation of difenoconazole residues in water

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and

<table>
<tr><th></th><th>Difenoconazole</th></tr>
<tr><td></td><td>would be observed in the chromatograms of the matrix control sample.</td></tr>
<tr><td>Calibration (type, number of data points)</td><td>Working solutions of difenoconazole at the concentrations 0.05, 0.1, 0.5, 1.0, 5.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</td></tr>
<tr><td>Calibration range</td><td>The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.
<table>
<tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr>
<tr><td>difenoconazole</td><td>126401</td><td>119.876</td><td>0.9999698</td></tr>
</table></td></tr>
<tr><td>Assessment of matrix effects is presented</td><td>Yes
Matrix effect was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration</td></tr>
<tr><td>Limit of determination/quantification</td><td>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:
<table>
<tr><th>Analyte</th><th>LOQ [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th><th>LOD [mg analyte/kg]</th><th>Equivalent calibration level [mg/L]</th></tr>
<tr><td>difenoconazole</td><td>0.001</td><td>0.1</td><td>0.0005</td><td>0.05</td></tr>
</table></td></tr>
</table>

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830 rev. 1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

A 2.1.2.7.1 Analytical method 1

A 2.1.2.7.1.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following report: Nina Schernikau, 2023
Report	Determination of the residues of difenoconazole and triazoles derivative metabolites in honey following application of Tores 250 EC in four trials in Northern Europe 2023, Nina Schernikau, 2023, Study code: SYT-2303
Guideline(s):	SANTE/2020/12830, rev. 2 SANTE/11956/2016, rev. 9
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for **difenoconazole** is based on the multi-residue method QuEChERS. In brief, for the analysis of difenoconazole samples of honey were extracted with acetonitrile after addition of water. The ratio was 10 mL of extraction solvent per g of matrix. Clean-up of the extract was performed by dispersive SPE with primary/secondary amine (PSA). The sample concentration in final extract was 0.025 g sample per mL of extract. Quantification was performed by use of LC-MS/MS detection.

The analytical method for **triazole derivative metabolites** is based on the QuPPe procedure. In brief, for triazole derivative metabolites (TDMs; 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA)) samples of honey were extracted with acidified methanol, after addition of water. The ratio was 2.0 mL of extraction solvent per g of matrix. An aliquot was transferred to a new tube, isotopically labelled internal standard(s) were added prior to evaporating the methanol phase of the extract. The water phase was further diluted using water. The sample concentration in final extract was 0.1 g sample per mL of extract. Quantification was performed by use of liquid chromatography coupled to differential mobility spectrometry – tandem mass spectrometry (LC-DMS-MS/MS) detection with isotopically labelled internal standard(s).

Analytical procedure - Difenoconazole

Reagents and materials

- Analytical procedure - Difenoconazole
- Reagents and solvents
- deionized water, Łukasiewicz-IPO
- acetonitrile (LC-MS), J.T. Baker, batch number 2114805856
- formic acid, ≥99% for LC-MS, VWR Chemicals, batch number DB634419
- anhydrous sodium sulphate (VI) pure p.a., J.T Baker, batch number 2106706810
- QuEChERS BEKOlut Citrate-Kit-01, BEKOlut GmbH & Co. KG, batch number 2821
- QuEChERS BEKOlut PSA-Kit-04, BEKOlut GmbH & Co. KG, batch number 4621

- QuEChERS BEKOlut PSA-Kit-08, BEKOlut GmbH & Co. KG, batch number 3621
- 99.8 ± 0.1 % standard of difenoconazole, Łukasiewicz-IPO Warsaw, Poland, batch number 2A/20
- standard solution of difenoconazole at the concentration of 1.0 mg/mL
- working solutions of difenoconazole at the concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 ng/mL

Preparation of standard solutions

Stock solution(s) of the analyte(s) were prepared by dissolving a weight of the reference item(s) with the aid of an ultrasonic bath. Each stock solution was allocated a unique reference number.
Solutions for fortification and calibration were obtained by (serial) dilution of the stock solution(s).
Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which were then fortified with intermediate solvent standard solutions.
All solutions were stored at typically 1 °C to 10 °C in a glass vial in the dark.

Sample Weight(s) and Fortifications

Control (untreated) samples were fortified prior to extraction with the fortification solutions as described below. The solvent was allowed to evaporate before starting the extraction procedure.

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level* (mg/kg)
Difenoconazole	Honey	1.0 ± 0.01	Z04	500	0.020	0.010
		1.0 ± 0.01	Z03	5000	0.020	0.10

Analytical procedure - Triazole Metabolites

Reagents and materials

- 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)

Preparation of standard solutions

Stock solution(s) of the analytes and isotopically labelled standards were prepared by dissolving a weight of the reference items with the aid of an ultrasonic bath. Each stock solution was allocated a unique reference number.
Solutions for fortification and calibration were obtained by (serial) dilution of the stock solution(s).
All solutions were stored at typically 1 °C to 10 °C in a glass vial in the dark.

Sample Weight(s) and Fortifications

Control (untreated) samples were fortified prior to extraction with the fortification solutions as described below.
The analytes were fortified jointly.

Fortified analyte(s)	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution used (ng/mL)	Volume of fortification solution added (mL)	Fortification level* (mg/kg)
1,2,4-	Honey	5.0 ± 0.05	Z112	100 (each)	0.50	0.010

Triazole, Triazole Alanine, Triazole Lactic Acid Triazole Acetic Acid		5.0 ± 0.05	Z111	1000 (each)	0.50	0.10

Results and discussions

Table A 13: Recovery results from method validation of difenoconazole and triazole metabolites using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Honey	Difenoconazole m/z 406→251*	0.01	108	8.3	-
		0.1	95	6.1	-
	Difenoconazole m/z 406→188	0.01	111	6.1	-
		0.1	104	4.5	-
	1,2,4-Triazole m/z 70→43 (Hypercarb)*	0.01	91	6.3	-
		0.1	91	4.1	-
	1,2,4-Triazole m/z 70→43 (Aquasil)	0.01	96	12	-
		0.1	88	5.9	-
	Triazole alanine m/z 157→70 (Hypercarb)*	0.01	88	3.4	-
		0.1	91	3.7	-
	Triazole alanine m/z 157→80 (Hypercarb)	0.01	88	8.1	-
		0.1	90	5.0	-
	Triazole lactic acid m/z 158→70 (Hypercarb)*	0.01	83	2.7	-
		0.1	87	4.9	-
	Triazole lactic acid m/z 158→70 (Aquasil)	0.01	94	2.7	-
		0.1	87	6.8	-
	Triazole acetic acid m/z 128→70 (Hypercarb)*	0.01	88	4.0	-
		0.1	86	3.9	-
	Triazole acetic acid m/z 128→70 (Aquasil)	0.01	84	13	-
		0.1	82	8.1	-

Table A 14: Characteristics for the analytical method used for validation of difenoconazole and triazole metabolites residues in honey

	Difenoconazole	Triazole metabolites
Specificity	The analyte difenoconazole was determined in the final sample extracts by use of LC-	The analytes 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid

	Difenoconazole	Triazole metabolites
	MS/MS detection with evaluation of one mass transition. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of target analyte. Untreated samples for accompanying control sample work up, for determination of (concurrent) recoveries and, if needed, for preparation of matrix-matched calibration standards were obtained from the local market. The blank values at the expected retention time of difenoconazole resulting from reagents and/or the control sample material used for recovery determinations and for preparation of matrix-matched calibration standards did not exceed a level that would correspond to 30 % of the LOQ. Correction for blank values was performed.	were determined in the final sample extracts by addition of isotopically labelled standards and by use of LC-DMS-MS/MS- detection. For triazole alanine, one mass transition was evaluated. A second mass transition was monitored for confirmation of peak identity but was not used for quantification of samples. For 1,2,4-triazole, triazole acetic acid and triazole lactic acid, one mass transition was evaluated. A confirmatory run was performed on a different stationary phase but was not used for quantification of samples. For each of the internal standards of 1,2,4-triazole, TA, TAA and TLA one mass transition was evaluated. Untreated samples for accompanying control sample work up and for determination of (concurrent) recoveries were obtained from the local market. No residues above 30 % of the LOQ were detected in the control (untreated) samples of honey used for recovery determinations. However, for the determination of residues of TDMs the recoveries were corrected by subtraction of control sample area from the respective recovery area.
Calibration (type, number of data points)	Minimum 5 levels of calibration.	
Calibration range	0.075 ng/mL to 7.5 ng/mL	0.30 ng/mL to 30 ng/mL
Assessment of matrix effects is presented	The effect of honey matrix on the detector response of difenoconazole was assessed by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at the same nominal concentrations. Matrix effects were < ± 20 % and deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.	Isotopically labelled internal standard was used for quantification of the triazole metabolites 1,2,4-triazole, TA, TAA and TLA so that possible matrix effects on the detector response are automatically compensated when using the response ratio of analyte to internal standard for quantification. Therefore, matrix effects on detection were not determined within this analytical phase.
Limit of determination/ quantification	LOQ = 0.01 mg/kg	LOQ = 0.01 mg/kg

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830, rev. 2 and fulfil its requirements.

A 2.1.2.7.2 Analytical method 2

A 2.1.2.7.2.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following report: Agnieszka Wojciech, MSc Eng., 2023
Report	SNS-F-11 Honeybees (*Apis mellifera L.*), Larval Toxicity Test, Repeated Exposure, Agnieszka Wojciech, 2023, Study code: B-01-23
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 1. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2.

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration with mixture of acetonitrile for HPLC and deionized water (50:50, v/v) (D1). The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0[1)]
1000 (Stock)	0.05	1	50.0[1)]
100.0	0.1	1	10.0[1)]
50.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]

1) Concentration level used for calibration. The range of linearity 1.0 mg difenoconazole/L to 100.0 mg difenoconazole/L.
D1 - mixture of acetonitrile for HPLC and deionized water (50:50, v/v)

Fortification samples

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	water	2	1.0	-	-	0.0
Fortification (LOQ)		5	1.0	100	0.1	10.0
Fortification (10x LOQ)		5	1.0	1000 (difenoconazole stock solution)	0.1	100.0

Sample preparation for the chromatographic analysis

Each sample in a minimum volume of 0.5 ml was diluted with acetonitrile in a ratio 1:1. The sample was diluted with a mixture of acetonitrile for HPLC and deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev.2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC grade	Łukasiewicz-IPO*	Fresh prepared before analysis	
Orthophosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC, LCMS	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025
		VMR Chemicals	22L294003	27.12.2025

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 1, 5, 10, 50 and 100 µg/mL in mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)

Equipment	Size, Description	Manufacturer/Supplier
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 2.6 µm C18 100Å 100 x 4.6 mm
Oven temperature	35°C
Injection Volume	1 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 15: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
water	difenoconazole	10.0	82.5	2.4	-
		100.0	78.8	2.9	-

Table A 16: Characteristics for the analytical method used for validation of difenoconazole residues in water

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix control sample.
Calibration (type, number of data points)	Working solutions of difenoconazole at the concentrations of 1.0, 5.0, 10.0, 50.0 and 100.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L. **Analyte** | **Slope** | **Intercept** | **Coefficient**

	Difenoconazole
	Difenoconazole | 5934.36 | -37.4216 | 0.9996501
Calibration range	The range of linearity of the analytical graph is from 1.0 mg/mL to 100.0 mg/mL. The range of calibration curve of difenoconazole is equivalent to range from 2.0 mg/L to 200.0 mg/L in water.
Assessment of matrix effects is presented	Yes Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analysed in addition.
Limit of determination/quantification	Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:

Analyte	**LOQ [mg analyte/L]**	**Equivalent calibration level [mg/L]**	**LOD [mg analyte/L]**	**Equivalent calibration level [mg/L]**
difenoconazole	10.0	5.0	2.0	1.0

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830 rev.1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev.2.

A 2.1.2.7.3 Analytical method 3

A 2.1.2.7.3.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following report: Agnieszka Wojciech, MSc Eng., 2023
Report	SNS-F-11 Bumblebees (*Bombus spp*.), Acute Oral Toxicity Test, Agnieszka Wojciech, 2023, Study code: B-02-23
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, Rev. 1.

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration with mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v) (D1). The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0[1)]
1000 (Stock)	0.05	1	50.0[1)]
100.0	0.1	1	10.0[1)]
100.0	0.05	1	5.0[1)]
10.0	0.1	1	1.0[1)]

1) Concentration level used for calibration. The range of linearity 1.0 mg difenoconazole/L to 100.0 mg difenoconazole/L.
D1 - mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v)

Fortification samples

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	sucrose solution	2	1.0	-	-	0.0
Fortification (LOQ)		5	1.0	100	0.2	20.0
Fortification (10x LOQ)		5	1.0	1000 (difenoconazole stock solution)	0.2	200.0

Sample preparation for the chromatographic analysis

First, 1 mL of sucrose solution was measured into a volumetric flask with a capacity of 10 mL and the volume was made up to 10 ml with mixture of acetonitrile for HPLC and deionized water (50:50; v/v). The sample was diluted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SAN-

TE/2020/12830 rev.2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC grade	Łukasiewicz-IPO*	Fresh prepared before analysis	
Orthophosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC, LCMS	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- 50% sucrose solution in water,
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,
- mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v),
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 1, 5, 10, 50 and 100 µg/mL in mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 2.6 µm C18 100Å 100 x 4.6 mm
Oven temperature	35°C
Injection Volume	1 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 17: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
sucrose solution	difenoconazole	20.0	103.5	1.4	-
		200.0	99.6	0.5	-

Table A 18: Characteristics for the analytical method used for validation of difenoconazole residues in sucrose solution

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix control sample.
Calibration (type, number of data points)	Working solutions of difenoconazole at the concentrations of 1.0, 5.0, 10.0, 50.0 and 100.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L. **Analyte** | **Slope** | **Intercept** | **Coefficient** Difenoconazole | 6056.19 | -188.048 | 0.9999850
Calibration range	The range of linearity of the analytical graph is from 1.0 mg/mL to 100.0 mg/mL. The range of calibration curve of difenoconazole is equivalent to range from 10.0 mg/L to 1000.0 mg/L in sucrose solution.
Assessment of matrix effects is presented	Yes Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analysed in addition.
Limit of determination/quantification	Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below: **Analyte** | **LOQ [mg analyte/L]** | **Equivalent calibration level [mg/L]** | **LOD [mg analyte/L]** | **Equivalent calibration level [mg/L]** difenoconazole | 20.0 | 2.0 | 10.0 | 1.0

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830, rev.1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2.

A 2.1.2.7.4 Analytical method 4

A 2.1.2.7.4.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following report: Agnieszka Wojciech, MSc Eng., 2023
Report	SNS-F-11 Bumblebees (*Bombus spp*.), Acute Contact Toxicity Test, Agnieszka Wojciech, 2023, Study code: B-03-23
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stock solution stability and limit of quantification and detection were determined. The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 1.

Sample preparation for the chemical determinations

Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration with mixture of 1% Triton(R)X100 solution and 50% acetonitrile for HPLC solution (1:9, v/v) (D1). The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0[1)]
1000 (Stock)	0.05	1	50.0[1)]
100.0	0.1	1	10.0[1)]

50.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]

1) Concentration level used for calibration. The range of linearity 1.0 mg difenoconazole/L to 100.0 mg difenoconazole/L.
D1 - mixture of 1% Triton(R)X100 solution and 50% acetonitrile for HPLC solution (1:9, v/v)

Fortification samples

For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	1% solution of triton in water	2	1.0	-	-	0.0
Fortification (LOQ)		5	1.0	100	0.2	20.0
Fortification (10x LOQ)		5	1.0	1000 (difenoconazole stock solution)	0.2	200.0

Sample preparation for the chromatographic analysis

Each sample in a minimum volume of 0.1 ml was diluted with mixture of acetonitrile for HPLC and deionized water (50:50; v/v) in a ration 1:9. The sample was diluted mixture of 1% Triton(R)X100 solution and 50% acetonitrile for HPLC solution (1:9, v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev.2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC grade	Łukasiewicz-IPO*	Fresh prepared before analysis	
Orthophosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC, LCMS	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025
Triton(R) X-100	-	Chempur	2012004087	12.2024

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,

- mixture of 1% Triton(R)X100 solution and 50% acetonitrile for HPLC solution (1:9, v/v),
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 1, 5, 10, 50 and 100 µg/mL in mixture of 1% Triton(R)X100 solution and 50% acetonitrile for HPLC solution (1:9, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 2.6 µm C18 100Å 100 x 4.6 mm
Oven temperature	35°C
Injection Volume	1 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (55 : 45, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 19: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
1% solution of triton in water	difenoconazole	20.0	107.0	3.7	-
		200.0	103.6	2.0	-

Table A 20: Characteristics for the analytical method used for validation of difenoconazole residues in 1% solution of triton in water

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix

<table>
<tr><th></th><th>Difenoconazole</th></tr>
<tr><td></td><td>control sample.</td></tr>
<tr><td>Calibration (type, number of data points)</td><td>Working solutions of difenoconazole at the concentrations of 1.0, 5.0, 10.0, 50.0 and 100.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded.

The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.

<table><tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td>Difenoconazole</td><td>5651.67</td><td>-3995.44</td><td>0.9995241</td></tr></table></td></tr>
<tr><td>Calibration range</td><td>The range of linearity of the analytical graph is from 1.0 mg/mL to 100.0 mg/mL. The range of calibration curve of difenoconazole is equivalent to range from 10.0 mg/L to 1000.0 mg/L in 1% solution of triton in water.</td></tr>
<tr><td>Assessment of matrix effects is presented</td><td>Yes
Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analysed in addition.</td></tr>
<tr><td>Limit of determination/quantification</td><td>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:

<table><tr><th>Analyte</th><th>LOQ [mg analyte/L]</th><th>Equivalent calibration level [mg/L]</th><th>LOD [mg analyte/L]</th><th>Equivalent calibration level [mg/L]</th></tr><tr><td>difenoconazole</td><td>20.0</td><td>2.0</td><td>10.0</td><td>1.0</td></tr></table></td></tr>
</table>

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830, rev.1 and fulfil its requirements. Validated method is also compatible to requirements of SANTE/2020/12830, rev. 2.

A 2.1.2.7.5 Analytical method 5

A 2.1.2.7.5.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference: Validation included in the following report:

	Agnieszka Wojciech, MSc Eng., 2023
Report	SNS-F-11 Honeybees (*Apis mellifera L.*), Chronic Oral Toxicity Test, Agnieszka Wojciech, 2023, Study code: B-04-23
Guideline(s):	SANTE/2020/12830 rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, accuracy, stock solution stability and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, rev. 2.

Sample preparation for the chemical determinations

Stock and standard solutions
The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solutions were prepared by dilution standards with a higher concentration with mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v) (D1). The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0
100.0	0.1	1	10.0
100.0	0.05	1	5.0[1)]
10.0	0.1	1	1.0[1)]
5	0.1	1	0.5[1)]
1	0.1	1	0.1[1)]
5	0.1	1	0.05[1)]

1) Concentration level used for calibration. The range of linearity 0.05 mg difenoconazole/L to 5.0 mg difenoconazole/L.
D1 - mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v)

Fortification samples
For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	sucrose solution	2	1.0	-	-	0.0
Fortification		5	1.0	100	0.01	1.0

(LOQ)						
Fortification (10x LOQ)		5	1.0	1000 (difenoconazole stock solution)	0.01	10.0

Sample preparation for the chromatographic analysis

First, 1 g of sucrose solution was measured into a volumetric flask with a capacity of 10 mL and the volume was made up to 10 mL with mixture of acetonitrile for HPLC and deionized water (50:50; v/v). The sample was diluted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SANTE/2020/12830 rev. 2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC grade	Łukasiewicz-IPO*	Fresh prepared before analysis	
Orthophosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023
Acetonitrile	HPLC, LCMS	POCH	0318/08/22	08.2025
		VMR Chemicals	22F274033	24.06.2025
			22L294003	27.12.2025

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- 50% sucrose solution in water,
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,
- mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v),
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions at concentration 0.05, 0.1, 0.5, 1, 5, 10 and 100 µg/mL in
- mixture of 50% sucrose solution and 50% acetonitrile for HPLC solution (1:9, v/v).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Balance	WPS 510/C	Radwag (Poland)
Balance	PS600.X2	Radwag (Poland)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)

Equipment	Size, Description	Manufacturer/Supplier
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 2.6 µm C18 100Å 100 x 4.6 mm
Oven temperature	35°C
Injection Volume	10 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 21: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
sucrose solution	difenoconazole	1.0	93.0	2.2	-
		10.0	97.6	0.4	-

Table A 22: Characteristics for the analytical method used for validation of difenoconazole residues in sucrose solution

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix control sample.
Calibration (type, number of data points)	Working solutions of difenoconazole at the concentrations of 0.05, 0.1, 0.5, 1.0 and 5.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L. **Analyte** / **Slope** / **Intercept** / **Coefficient** Difenoconazole / 59375.6 / 135.947 / 0.9999832

<table>
<tr><th></th><th>Difenoconazole</th></tr>
<tr><td>Calibration range</td><td>The range of linearity of the analytical graph is from 0.05 mg/mL to 5.0 mg/mL. The range of calibration curve of difenoconazole is equivalent to range from 0.5 mg/L to 50.0 mg/L in sucrose solution.</td></tr>
<tr><td>Assessment of matrix effects is presented</td><td>Yes
Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analyzed in addition.</td></tr>
<tr><td>Limit of determination/quantification</td><td>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).

Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below:
<table>
<tr><th>Analyte</th><th>LOQ [mg analyte/L]</th><th>Equivalent calibration level [mg/L]</th><th>LOD [mg analyte/L]</th><th>Equivalent calibration level [mg/L]</th></tr>
<tr><td>difenoconazole</td><td>1.0</td><td>0.1</td><td>0.5</td><td>0.05</td></tr>
</table>
</td></tr>
</table>

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830, rev. 2 and fulfil its requirements.

A 2.1.2.7.6 Analytical method 6

A 2.1.2.7.6.1 Method validation

Comments of zRMS:	Method is accepted for the generation of pre-authorization data

Reference:	Validation included in the following reports:
Report	SNS-F-11 Terrestrial Plant Test: Vegetative Vigour Test, Paweł Pieczka, MSc Eng., 2024, Study code: G-46-24 SNS-F-11 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, Aneta Gierbuszewska, MSc, 2024, Study code: G-47-24
Guideline(s):	SANTE/2020/12830 rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The aim of the analytical part was to (i) validate the analytical method (ii) determine concentrations of active substance of test item in the test samples.
The validation of analytical method was performed according to SANTE/2020/12830, Rev. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, accuracy, stock solution stability and limit of quantification and detection were determined.

Sample preparation for the chemical determinations

Stock and standard solutions
The stock solution with a concentration of 1.0 mg/mL were prepared by weighting 10.0 mg of standard into a volumetric flask with a capacity of 10.0 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10.0 mL with the same solvent. The working solution of difenoconazole with a concentration of 50 and 100 µg/mL was prepared by dilution of the stock solution with mixture of acetonitrile for HPLC and deionized water (50:50, v/v). The working solutions were prepared by dilution standards with a higher concentration. The dilutions were made as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000 (Stock)	0.1	1	100.0[1)]
1000 (Stock)	0.05	1	50.0[1)]
100.0	0.1	1	10.0[1)]
50.0	0.1	1	5.0[1)]
10.0	0.1	1	1.0[1)]

1) Concentration level used for calibration. The range of linearity 1.0 mg difenoconazole/L to 100.0 mg difenoconazole/L.
D1 - mixture of acetonitrile for HPLC and deionized water (50:50, v/v)

Fortification samples
For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solution. The appropriate amount of spiking solutions was added to the matrix to prepare LOQ and 10xLOQ. Samples were prepared as exemplarily described in the table below:

Sample Type	Matrix	Number of Replicates	Sample [mL]	Concentration of Spiking Solution [mg/L]	Volume Of Spiking Solution [mL]	Level of Fortification [mg/L]
Control	deionized water	2	1.0	-	-	0.0
Fortification (LOQ)		5	1.0	100	0.1	10.0
Fortification (10x LOQ)		5	1.0	1000	0.1	100.0

Sample preparation for the chromatographic analysis
Each sample in a minimum volume of 0.5 mL was diluted with acetonitrile for HPLC in a ration 1:1. The sample was diluted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SAN-

TE/2020/12830 rev. 2.

Conditions of the chemical determinations

Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC grade	Łukasiewicz-IPO*	Fresh prepared before analysis	
Orthophosphoric acid	85% HPLC	Honeywell, Fluka	M2520	29.08.2024
Acetonitrile	HPLC	CHEMPUR	240202058	02.2026

* The main stages of water purification: pre-treatment (mechanical filter, activated carbon), deionization (ion exchange resin). Water prepared with SolPure-7 water deionizer

Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile for HPLC and deionized water (50:50, v/v) i.e. 50% acetonitrile for HPLC solution,
- standard solution of 1 mg/mL of difenoconazole in acetonitrile for HPLC,
- working solutions of difenoconazole at concentration 100.0, 50.0, 10.0, 5.0 and 1.0, µg/mL in mixture of acetonitrile for HPLC and deionized water (50:50, v/v) (matrix matched standards).

Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Chromatograph	Prominence	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

	Parameter
Chromatographic System	High Performance Liquid Chromatography (HPLC)
Chromatograph	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical Column	Kinetex 2.6 µm C18 100Å 100 x 4.6 mm
Oven temperature	35°C
Injection Volume	1 µL
Mobile Phase	acetonitryl HPLC : ortho-phosphoric acid solution 0.05 % (60 : 40, v/v)
Flow Rate	0.70 mL/min
Wave length	220 nm
Detection System	Diode Array Detector

Table A 23: Recovery results from method validation of difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
deionized water	difenoconazole	10.0	97.0	1.0	-
		100.0	99.9	0.9	-

Table A 24: Characteristics for the analytical method used for validation of difenoconazole residues in deionized water

	Difenoconazole
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance was overlapping with matrix signal of the control samples in the experiments conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference are directly apparent and would be observed in the chromatograms of the matrix control sample.
Calibration (type, number of data points)	Working solutions of difenoconazole at the concentrations of 1.0, 5.0, 10.0, 50,0 and 100.0 mg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The equations of the calibration line were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L. **Analyte** | **Slope** | **Intercept** | **Coefficient** Difenoconazole | 5715.45 | 459.001 | 0.9999545
Calibration range	The range of linearity of the analytical graph is from 1.0 mg/mL to 100.0 mg/mL. The range of calibration curve of difenoconazole is equivalent to range from 1.0 mg/L to 100.0 mg/L in deionized water.
Assessment of matrix effects is presented	Yes Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analysed in addition.
Limit of determination/quantification	Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%). Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below: **Analyte** | **LOQ [mg analyte/L]** | **Equivalent calibration level [mg/L]** | **LOD [mg analyte/L]** | **Equivalent calibration level [mg/L]** difenoconazole | 10.0 | 5.0 | 2.0 | 1.0

Conclusion

The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation. The validated analytical methods was performed according to SANTE/2020/12830, rev. 2 and fulfil its requirements.