

FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: IN005B1570

Product name: ~~INDOFIL~~ Difenoconazole 250 G/L EC greener

Chemical active substance:

Difenoconazole, 250 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(Article 33: Application for authorisation)

Applicant: Indofil Industries (Netherlands) B.V.

Submission date: January 2022

MS Finalisation date: 10.2022 ; 11.2023 05.2024 08 2024

Version history

When	What
January 2022	V0 – Original version from applicant Indofil Industries (Netherlands) B.V. for submission to z-RMS, Poland, in the frame of the PPP Authorization according to Article 33 of Regulation (EC) No. 1107/2009
October 2022	zRMS first evaluation
October 2023	Applicant inclusion of final reports of studies underway at time of original submission
November 2023	Assessment of additional data provided by the Applicant
February 2024	Applicant dRR update commenting period
March 2024	Additional applicant dRR update based on cMS cmmnts
May 2024	Assessment of the updated data
June 2024	Applicant update to include chronic laboratory studies with adult honey bees and honey bee larvae
August 2024	Assessment in relation to third round of comments and applicant update

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5 Analytical methods

This document reviews the analytical methods for the product IN005B1570, an emulsifiable concentrate formulation containing 250 g/L difenoconazole for use on oilseed rape, pome fruits, carrot, cauliflower, broccoli and cabbage. Difenoconazole was first included in Annex I to Directive 91/414/EEC by Commission Directive 2008/69/EC of 1 July 2008.

A full risk assessment according to Uniform Principles is provided which demonstrates that the product is safe for the environment. Where appropriate this document refers to the conclusion of the EU review for difenoconazole. This will be where:

- The active substance data are relied upon in the risk assessment of the formulation; or when
- the EU review concluded that the additional data/information should be considered at national re-registration.

Note: this Part B document only reviews data (Annex II or Annex III) and additional information that has not previously been considered within the EU review process, as part of the Annex I inclusion decision. New annex II data must only be included if they are considered essential for the evaluation and in this case a full study summary must be provided. In the case where the formulation has been previously evaluated, at European level, detailed summaries have not been provided.

This product was not the representative formulation and has not been previously evaluated according to the Uniform Principles.

The EFSA Scientific report for Difenoconazole (EFSA Scientific Report, 2011; 9(1):1967) is considered to provide the relevant review information or a reference to where such information can be found.

The Commission Implementation Regulation for Difenoconazole (540/2011) provides specific provisions under part B which need to be considered by the applicant in the preparation of their submission and by the MS prior to granting an authorisation.

For the implementation of the uniform principles as referred to in Article 29(6) of Regulation (EC) No. 1107/2009, the conclusion of the review report for Difenoconazole, and in particular Appendices I and II thereof, as finalised in the Standing Committee on the Food Chain and Animal Health shall be taken into account.

In this overall assessment Member States must pay particular attention to:

- The protection of aquatic organisms.

Conditions of use shall include adequate risk mitigation measures, where appropriate.

The Commission Implementation Regulation (1100/2011) amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of Difenoconazole provides specific provisions under part B which need to be considered by the applicant in the preparation of their submission and by the MS prior to granting an authorisation.

For the implementation of the uniform principles, as referred to in Article 29(6) of Regulation (EC) No 1107/2009, the conclusions of the review report on difenoconazole, and in particular Appendices I and II thereof, as finalised in the Standing Committee on the Food Chain and Animal Health on 27 September 2011 shall be taken into account.

In this overall assessment Member States shall pay particular attention to the protection of aquatic organisms.

Conditions of use shall include adequate risk mitigation measures, where appropriate.

The notifier shall submit confirmatory information as regards:

- (a) further data on the specification of the technical material;
- (b) residues of triazole derivative metabolites (TDMs) in primary crops, rotational crops, processed commodities and products of animal origin;
- (c) the potential for endocrine disrupting effects on fish (fish full life cycle study) and the chronic risk to earthworms from the active substance and the metabolite CGA 205375 (1);
- (d) the possible impact of the variable isomer-ratio in the technical material and of the preferential degradation and/or conversion of the mixture of isomers on the worker risk assessment, the consumer risk assessment and on the environment.

The notifier shall submit to the Member States, the Commission and the Authority the information set out in point (a) by 31 May 2012, the information set out in points (b) and (c) by 30 November 2013 and the information set out in point (d) within 2 years from the adoption of specific guidance.'

Information on the detailed composition of IN005B1570 can be found in the confidential dossier of this submission (Registration Report – Part C).

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are available for the active substance(s) Difenoconazole and relevant impurities Toluene in the plant protection product Difenoconazole 250 g/L EC Greener. Noticed data gaps are:

- None

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

Noticed data gaps are:

- An 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 in Poland – after renewal of active substance).
- An analytical method for difenoconazole in body fluids is required according to Regulation (EC) No 283/2013 (post registration requirement in Poland – after renewal of active substance)
- Information on analytical methods for determining difenoconazole residues in animal matrices compliant with SANTE/2020/12830 rev. 2 (post registration requirement in Poland – after renewal of active substance).

Deadline for completion all above mentioned requirements may be considered at the Member State level (pre or post registration requirements).

Commodity/crop	Supported/ Not supported
Oilseed rape	Supported
Apple	Supported
Carrot	Supported
Tomato	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)

Analytical methods for the analysis of difenconazole in the plant protection product IN005B1570 have not been previously evaluated within a peer reviewed process at EU level. An overview on the acceptable methods and possible data gaps for analysis of difenconazole in the plant protection product IN005B1570 is provided as follows:

Comments of zRMS:	Accepted. The analytical method provided is validated and meets criteria of specificity, linearity and precision according to the requirements of SANCO 3030/99 rev. 5.
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Reference:	KCP 5.1.1/01
Report	Difenconazole 250 g/L EC Greener – IN005B1570: Validation of the Analytical Method for the Determination of the Active Ingredient Content. Urbani, M. 2021 ChemService Study No. 0330/2021
Guideline(s):	Yes. - SANCO/3030/99 rev. 5 dated 22/03/2019
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Difenconazole content in ~~INDOFIL~~–Difenconazole G/L 250 EC Greener is quantified using HPLC-UV/Vis. Test material is dissolved in acetonitrile, filtered and analysed without further processing. Concentration is determined against an external calibration range.

In order to demonstrate the validity of the analytical method, the following validity criteria have to be respected:

Specificity / Interference: for a.s. interference not >3% of total peak area for target analyte.

Linearity: Calibration appropriate to the nominal concentration range at least $\pm 20\%$ of relevant analytical solutions

- duplicate determinations at 3 concentrations, or
- single determinations at 5 concentrations.

Repeatability (Precision):

Minimum of 5 replicate sample determinations.

Acceptability criteria: Horrat value < 1

Recovery (Trueness):

At least 2 independent recovery determination (two weights).

Each recovery value in the range 97 - 103 % for active ingredient content $\geq 10\%$ w/w.

Chromatographic conditions

HPLC column

Phenomenex or equivalent	:	Luna Phenyl-Hexyl 100 Å, 5 µm, 250 x 4.6 mm i.d.
Detector	:	244 nm
Column temperature	:	20°C
Eluent A	:	Water
Eluent B	:	Acetonitrile
Eluent D	:	Phosphoric acid at 10 % v/v
Gradient	:	A:B:D 35:55:10
Eluent flow	:	1.0 mL/min
Volume of injection	:	10 µL
Difenoconazole Retention time	:	about 14.4 minutes
Total Analysis Time	:	20 minutes

Description of the method validation

Specificity

The specificity test was conducted injecting, in the adjusted chromatographic conditions, the following samples, comparing the chromatograms in order to check possible cross contaminations.

Injected solution samples	Nominal injected Concentration (µg/mL)
Blank (acetonitrile)	0
Difenoconazole reference material	100
Difenoconazole technical test substance	100
Placebo	0
Test item	100
Fortified Placebo	100

Linearity

Linear regression analysis was performed using the least squares method.

The correlation coefficient was calculated using regression analysis.

Preparation of the stock reference material solution and stock internal standard solution

Using the analytical balance, the volumetric flask and the volumetric pipette, stock reference material solution was prepared in acetonitrile as follows:

Reference material	Stock reference material solution (SRMS)			
	Weight (mg) ¹	Purity (%)	Total volume (mL) ²	Concentration (µg/mL)
Difenoconazole	20.8	95.5	20.00	993.20

(1) Weight of the reference material.

(2) Total volume of the stock reference material solution

Preparation of the working standard solutions

Using volumetric flasks and volumetric pipettes, five working standard solutions for linear calibration were prepared in acetonitrile as follows:

Working Standard Solution	Stock reference material solution (mL)	Final Volume (mL)	Concentration (µg/mL)	Linearity range (g/L) ¹
Blank	0	10.00	0	-
WSS 1	0.50	10.00	49.66	125.82-377.45
WSS 2	0.75	10.00	74.49	
WSS 3	1.00	10.00	99.32	
WSS 4	1.23	10.00	124.15	
WSS 5	1.50	10.00	148.98	

(1) Calculated with respect to the nominal test item weight and preparative in repeatability and considering the density value 1.1401 g/mL determined in the CH - 0329/2021 GLP study.

After the injection of the working standard solutions, from the lowest to the highest concentration, a solvent wash was also injected in order to verify if memory peaks were detected.

Repeatability (Precision)

Five solutions of the test item (labelled from A to E) were prepared and injected as detailed in Internal Analytical Method No. 0330/2021.

Preparation of stock reference material solution:

The stock reference material solution and relevant working standard solutions are the same already prepared for Linearity.

Preparation of test item solutions:

450 mg of the sample is weighed into a 100 mL volumetric flask, and the flask filled to the mark using acetonitrile. Using a volumetric pipette, 1.00 mL of the stock is solution is transferred into a 10.00 mL volumetric flask and made up to volume with acetonitrile. An aliquot of the diluted test item solution was transferred into a vial for the HPLC analysis. The summary of test item preparation procedure is presented in the following table:

	Stock test item solution (STIS)		Diluted test item solution (DTIS)	
	Nominal weight (mg)	Volume (mL)	Taken volume (mL) ¹	Total volume (mL) ²
Test item	450	100.00	1.00	10.00

(1) Volume taken from stock test item solution

(2) Total volume of diluted test item solution.

Precision of the analytical method was assessed with the data obtained.

Recovery (Trueness)

The test was performed by spiking two aliquots of the Placebo with the Difenoconazole test substance, corresponding to additions of 100 % of the nominal concentration of active ingredient.

Preparation of stock reference material solution:

The stock reference material solution and relevant working standard solutions are the same already prepared for Linearity.

Preparation of the fortified placebo solutions

Using the analytical balance, the fortified Placebo solutions were prepared in acetonitrile as follows

	Placebo nominal weight (mg)	Difenoconazole test substance nominal weight (mg)
Spike	348.7	100

The whole procedure was repeated for fortified samples. The tests item solutions were analysed by HPLC/UV.

Recovery of the analytical method was assessed with the data obtained.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance Difenoconazole in plant protection product Difenoconazole 250 g/L EC Greener

	Difenoconazole	Validity Criteria
Author(s), year	M. Urbani, 2021a	
Principle of method	HPLC-UV/Vis	
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	Range: 49.66 µg/mL – 148.98 µg/mL, corresponding to Difenoconazole nominal content in formulation: 125.82 – 377.45 g/L $y = 1.48x + 1.43$ $r = 0.99974$ (n = 5)	Correlation coefficient $r > 0.99$
Precision – Repeatability Mean n = 5 (%RSD)	RSD = 1.30 % At 22.4 % w/w (256 ± 3 g/L) RSDr = 1.68 Hr = 0.77	$Hr \leq 1$
Accuracy n = 2 (% Recovery)	Spike A = 100.43 % Spike B = 98.00 % Mean recovery: 99.21 %	97 – 103 %
Interference/ Specificity	No interfering peaks observed.	Interference < 3 %
Comment		

Conclusion

The analytical methods for the determination of active substance difenoconazole in the plant protection product ~~INDOFIL~~ Difenonazole 250 G/L EC Greener has been described and validated according with SANCO/3030/99 rev. 5.

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

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

Comments of zRMS:	Accepted. The analytical method for Toluene is validated and meets criteria of specificity, linearity and precision according to the requirements of SANCO 3030/99 rev. 5.
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Reference:	KCP 5.1.1/02
Report	Difenoconazole 250 g/L EC Greener – IN005B1570: Validation of the Analytical Method for the Determination of Toluene as Relevant Impurity Content. Urbani, M. 2021 ChemService Study No. 0331/2021
Guideline(s):	Yes - SANCO/3030/99 rev. 5 dated 22/03/2019
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The identity of Toluene content in Difenoconazole 250 g/L EC Greener – IN005B1570 is confirmed using GC/FID. Test material is dissolved in acetone, filtered and analysed without further processing. Concentration is determined against an external calibration range.

In order to demonstrate the validity of the analytical method, the following validity criteria have to be respected:

Specificity / Interference: for a.s. interference not >3% of total peak area for target analyte.

Linearity: Calibration appropriate to the nominal concentration range at least $\pm 20\%$ of relevant analytical solutions

- duplicate determinations at 3 concentrations, or
- single determinations at 5 concentrations.

LOQ: Lowest fortification level

Repeatability (Precision):

Minimum of 5 replicate sample determinations.

Acceptability criteria: Horrat value < 1

Recovery (Trueness):

At least 2 independent recovery determination (two weights).

Each recovery value in the range:

70 - 130 % for content < 0.01 % w/w;

75 - 125 % for content \geq 0.01 % w/w and < 0.1 % w/w;

80 - 120 % for content \geq 0.1 % w/w and < 1 % w/w;

90 - 110 % for content \geq 1 % w/w and < 10 % w/w;

97 - 103 % for content \geq 10 % w/w

Chromatographic conditions:

GC column	J. & W. (Agilent) or equivalent HP-5, 30 m x 0.32 mm I.D., film thickness 0.25 μ m
Liner	Inlet liner, splitless, single taper, glass wool, deactivated. Part Number 5062-3587
Detector	FID
Oven program	Initial 40°C for 1 min. 10°C/min. from 40°C to 100°C 0 min. at 100°C; 40°C/min. from 100°C to 300°C 15 min. at 300°C.
Injector temperature	220°C
Detector temperature	200 °C
Volume of injection	1 μ L
Carrier gas	He
Flow	2 mL/min
Inlet mode	Split
Total flow	18.333 mL/min.
Split flow	13.333 mL/min.
Split ratio	6.6667:1
Pressure	63.043 kPa (at 40°C)
Toluene ret. time	about 3.3 minutes
Total analysis time	22 minutes

Description of the method validation

Specificity

The specificity test was conducted injecting, in the adjusted chromatographic conditions, the following samples, comparing the chromatograms in order to check possible cross contaminations.

Injected solution samples	Nominal injected Concentration (μ g/mL)
Wash (acetone)	-
Toluene reference material	2.50
Test item	-
Test item fortified at low level	-
Test item fortified at high level	-

Confirmatory

The identity of Toluene relevant impurity in the test item was conducted injecting, in the GC/MS adjusted chromatographic conditions, the following samples, comparing the chromatograms in order to check possible cross contaminations.

Injected solutions	Nominal injected concentration (µg/mL)
Wash (acetone)	-
Toluene reference material	2.50
Test item	-
Test item fortified at low level	-
Test item fortified at high level	-

Chromatographic conditions GC/MS/FID for confirmatory test

GC column	Phenomenex or equivalent ZB-5MS UI, 30 m x 0.25 mm I.D., film thickness 0.25 µm
Detector	FID and EI MSD
Oven program	Initial 40°C for 1 min. 10°C/min. from 40°C to 100°C 40°C/min. from 100°C to 300°C 15 min. at 300°C.
Injector temperature	220°C
Volume of injection	2 µL
Carrier gas	He
Flow	2.1 mL/min
Inlet mode	Splitless
Pressure	21.869 psi (at 40°C)
Toluene ret. time	about 3.8 minutes
Total analysis time	25 minutes
FID conditions	
Detector temperature	300°C
H2 Flow	40 mL/min
Air Flow	400 mL/min
Makeup Flow	25 mL/min
EI MSD conditions	
Acquisition mode	Scan (from 30.0 to 500.0)
Solvent delay	2.00 min
EM Voltage	1553
MS source	230°C
MS Quadrupole	150°C
MS OFF	from 6.0 minutes to 25.00 minutes

Linearity

Linear regression analysis was performed using the least squares method.
The correlation coefficient was calculated using regression analysis.

Preparation of the stock reference material solution and stock internal standard solution

Using the analytical balance, the volumetric flask and the volumetric pipette, stock reference material solution was prepared in acetone:

Reference material	Stock reference material solution (SRMS)			Diluted reference material solution (DRMS)		
	Weight (mg) ¹	Total volume (mL) ²	Concentration (µg/mL)	Taken volume (mL) ³	Total volume (mL) ⁴	Concentration (µg/mL)
Toluene	22.5	20.00	1123.88	1.25	50.00	28.10

- (1) Weight of the reference material (99.9% purity)
(2) Total volume of the stock reference material solution
(3) Volume taken from the stock reference material solution
(4) Total volume of diluted reference material solution

Preparation of the working standard solutions

Using volumetric flasks and volumetric pipettes, five working standard solutions for linear calibration were prepared in acetone:

Working Standard Solution	Stock reference material solution (mL)	Final Volume (mL)	Concentration (µg/mL)	Linearity range (µg/g) ¹
Blank	0	10.00	0	-
WSS 1	0.10	10.00	0.28	56.19-5619.38
WSS 2	0.50	10.00	1.40	
WSS 3	1.00	10.00	2.81	
WSS 4	2.00	10.00	5.62	
WSS 5	10.00	10.00	28.10	

- (1) Calculated with respect to the nominal test item weight and preparative in repeatability.

After the injection of the working standard solutions, from the lowest to the highest concentration, a solvent wash was also injected in order to verify if memory peaks were detected.

Limit of Detection and Limit of Quantification

The limit of detection (L.O.D.), defined as half the lowest working standard solution WSS 1, was a final injected solution of 0.13 µg/mL, corresponding to 25.00 µg/g for the relevant impurity in the test item. The limit of quantification (L.O.Q.), defined as the lowest fortification level, was a final injected solution of about 0.50 µg/mL for the impurity, corresponding to about 100.00 µg/g in the test item.

Relevant impurity	L.O.D. (µg/mL) ¹	L.O.D. (µg/g) ²	L.O.Q. (µg/mL) ¹	L.O.Q. (µg/g) ²
Nominal values	0.13	25.00	0.50	100.00
Toluene	0.14	28.10	0.56	112.39

- (1) Injected concentration
(2) Calculated respect to the test item.

Impurity results calculated as < 25.00 µg/g (L.O.D.) are classified as not detected (n.d.).

Impurity results calculated as greater than the limit of detection but less than the limit of quantification, are designated as < 100.00 µg/g.

If an impurity content is calculated as greater than 5000 µg/g in the test item, the test item solution must be suitably diluted using volumetric glassware

Repeatability (Precision)

Five solutions of the test item (labelled from A to E) were prepared and analysed as detailed in Internal Analytical Method No. 0331/2021.

Preparation of stock reference material solution:

The stock and diluted reference material solutions and relevant working standard solutions are the same already prepared for Linearity.

Preparation of test item solutions:

Using the analytical balance, about 250 mg of the test item were weighed into a 50.00 mL volumetric flask, dissolving to volume with acetone. An aliquot of the stock test item solution was then transferred into a vial for the GC/FID analysis. The summary of test item preparation procedure is presented in the following table:

	Stock test item solution (STIS)	
	Nominal weight (mg)	Volume (mL)
Test item	250	50.00

Precision of the analytical method was assessed with the data obtained.

Recovery (Trueness)

The test item was spiked five times at low level and two times at high level with the fortification reference material solution (FRMS) corresponding to the following impurity additions.

Preparation of stock reference material solution:

The stock reference material solution and relevant working standard solutions are the same already prepared for Linearity.

Relevant impurity	Low level (µg/g)	High level (µg/g)
Toluene	112.39	1123.88

The stock and diluted reference material solutions and relevant working standard solutions are the same already prepared for Linearity

Preparation of the stock and diluted fortification reference material solutions

Using the analytical balance and a volumetric flask, a stock reference material solution was prepared in acetone as follows

Reference material	Stock reference material solution (SRMS)		
	Weight (mg) ¹	Total volume (mL) ²	Concentration (µg/mL)
Toluene	22.5	20.00	1123.88

(1) Weight of the reference material (99.9% purity)

(2) Total volume of the stock reference material solution

Using volumetric flasks and volumetric pipettes, two diluted fortification reference material solutions were prepared in acetone as follows

Reference material	1 st Diluted fortification reference material solution (1DFS)			2 nd Diluted fortification reference material solution (2DFS)		
	Taken volume (mL) ¹	Total volume (mL) ²	Concentration (µg/mL)	Taken volume (mL) ³	Total volume (mL) ⁴	Concentration (µg/mL)
Toluene	5.00	10.00	561.94	1.00	10.00	56.19

(1) Volume taken from the stock fortification reference material solution

(2) Total volume of 1st diluted fortification reference material solution

(3) Volume taken from the 1st diluted fortification reference material solution

(4) Total volume of 2nd diluted fortification reference material solution

Preparation of the fortified test item solutions

Using volumetric flasks and volumetric pipettes, fortified test item solutions at Low and High level were prepared in acetone as follows

	Five Spike Low (~ 100 mg/kg)	Two Spike High (~ 1000 mg/kg)
Test item (mg)	250	250
1DFS (mL)	-	0.50
2DFS (mL)	0.50	-
Total volume (mL)	50.00	50.00

Recovery of the analytical method for each impurity was assessed with the data obtained.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) Difenoconazole 250 g/L EC Greener – IN005B1570

	Toluene max. content in PPP	Validity Criteria
Author(s), year	Urbani, M. 2021b	
Principle of method	GC/FID	
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	Range: 0.28 µg/mL to 28.10 µg/mL 56.19 µg/g to 5619.38 µg/g (Toluene nominal content in the test item) $y = 82009x + 16011$ $r = 0.99997$ (n=5)	Correlation coefficient $r > 0.99$
LOQ	100.00 µg/g (or 0.50 µg/mL injected)	Lowest fortification level
LOD	25.00 µg/g (or 0.13 µg/mL injected)	-
Precision – Repeatability Mean n = 5 (%RSD)	RSD = 3.05% At 112.39 µg/g RSDr = 5.37 Hr = 0.57	$Hr \leq 1$
Accuracy (% Recovery)	Low level, n = 5 (96.5%) High level, n = 2 (111.4%)	75 - 125 % for content ≥ 0.01 % w/w and < 0.1 % w/w; 80 - 120 % for content ≥ 0.1 % w/w and < 1 % w/w;
Interference/ Specificity	Interference from impurities in a.s. not >3% of total peak area for target analyte.	Interference < 3 %

	Toleune max. content in PPP	Validity Criteria
Comment		

Conclusion

The analytical methods for the determination of impurity toluene in the plant protection product Difenoconazole 250 g/L EC Greener – IN005B1570 has been described and validated according with SANCO/3030/99 rev. 5.

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

There are no formulants or constituents of formulants within the preparation or formed during storage, that are of toxicological, ecotoxicological or environmental relevance. Therefore, this point is not relevant.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are not CIPAC available methods for the quantification of difenoconazole in emulsifiable concentrate applicable to Difenoconazole 250 g/L EC Greener – IN005B1570.

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
Plants, plant products,... (Residues)	Primary	0.1 mg/kg Apple, lettuce, wheat (grain), oil seed rape	LC-MS/MS	Steinhauser S. 2004a (primary) Schulz H. 2004 (ILV) EU agreed
	Primary	0.05 mg/kg Tomatoes, potaties	GC-NPD	Williams W.L., Shoffner K.P. 1987 EU agreed
	Primary	0.05 mg/kg wheat raw agric.	GC-NPD	Williams W.L. 1988 Whetzel J.E. 1990 EU agreed
	Primary	0.01 mg/kg Wheat raw agric., apple/pear, carrot	GC-NPD	Ross J. 1991 EU agreed

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
	Primary	0.02 mg/kg carrot	GC-ECD	Bussy L., Maffexxoni M. 1993 EU agreed
	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC-MS/MS	Longhi, D. 2021a (KCP 5.1.2-01)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC-MS/MS	Longhi, D. 2021b (KCP 5.1.2-02)
	ILV (dried apples)			Rigamonti E. 2022a (on-going) (KCP 5.1.2-03)
	ILV (dried tomatoes)			Rigamonti E. 2022b (on-going) (KCP 5.1.2-04)
Animal products, food of animal origin,... (Residues)	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat, milk and hen eggs	GC-NPD	Crook S.J. 2004 (primary) Ryan J. 2004b Benazerat L. 2004 (ILV) EU agreed
	Primary	0.01 mg/kg Milk 0.05 mg/kg Bovine liver, kidney, muscle, fat and hen eggs	LC-MS/MS	Wurx R.E.M. 1994 EU agreed
	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat 10 µg/l blood 5 µg/l Milk	LC-MS/MS	Tribolet, R. 2000 EU agreed
Soil, water, sediment,... (Environmental fate)	Primary	0.01 mg/kg	LC-MS/MS	Tummon O.K. 2004a EU agreed
	Primary	0.04-0.05 mg/kg silty clay loam, everglades peat, silt loam, sand, clay, sandy loam	GC-NPD GC-ECD	Williams, R.K. 1986 Kuhne-Thu H. 1990b Kuhne-Thu H. 1990a EU agreed
	Primary	0.04 mg/kg overall soil	GC-ECD	Williams, R.K and Shoffner, K.P. 1987 Kuhne-Thu H. 2000 Kuhne-Thu H. 1990c Kuhne-Thu H. 1990d Kuhne-Thu H. 1991a Kuhne-Thu H. 1991b Kuhne-Thu H. 1990b

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
				Kuhne-Thu H. 1990a EU agreed
	Primary	0.02 mg/kg sandy loam and loam	GC-ECD	Ross, J. 1991 Kuhne-Thu H. 2000 Kuhne-Thu H. 1992a EU agreed
	Primary	0.05 mg/kg overall soil	GC-ECD	Kuhne-Thu H. 1996 Tack T. 1995 EU agreed
	Primary	0.05-0.1 µg/l surface water and drinking water (potable)	GC-ECD	Tribolet, R. 1999a Tribolet, R. 1999b EU agreed
	Primary	0.1 µg/l HPLC-grade and drinking water (potable)	GC-ECD	Tribolet, R. 1990 EU agreed
Soil, water,... (Efficacy)	Primary	--	--	--
	Confirmatory (if required)	--	--	--
Feed, body fluids,... (Toxicology)	Primary	--	--	Difenoconazole is not classified as toxic or highly toxic, therefore no analytical method is required
	Confirmatory (if required)	--	--	Difenoconazole is not classified as toxic or highly toxic, therefore no analytical method is required
Soil, water,... (Ecotoxicology)	Primary and confirmatory	42.3 µg/kg soil	HPLC-MS triple quadrupole detector	Garagna, D. 2021a (KCP 5.1.2-11)
	Primary and confirmatory	0.23 mg/L Stock Solutions	HPLC-MS triple quadrupole detector	Garagna, D. 2021b (KCP 5.1.2-12)
	Primary and confirmatory	9.5 µg/L - 9.6 µg/L aqueous samples	HPLC-MS triple quadrupole detector	Garagna, D. 2021c (KCP 5.1.2-13)
	Primary and confirmatory	6.04 mg/L in sucrose solution 0.06 mg/L in water HPLC grade	HPLC-MS/MS	Tediosi, E. 2023c (KCP 5.1.2-16)
Air	Primary	0.99 ng/l	LC-MS/MS	Tummon O.J. 2004b EU agreed

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
	Primary	0.1 µg/l	GC-ECD	Tribolet, R. 1992 Tribolet, R. 1996 EU agreed
Aqueous samples	Primary and confirmatory	9.5 µg/L - 9.6 µg/L aqueous samples	HPLC-MS triple quadrupole detector	Garagna, D. 2021c (KCP 5.1.2-13)
	Primary and confirmatory	6.04 mg/L in sucrose solution 0.06 mg/L in water HPLC grade	HPLC-MS/MS	Tediosi, E. 2023c (KCP 5.1.2-16)

Table 5.2-4: Validated methods for the generation of pre-authorization data

Component of residue definition: Metabolite CGA 205375				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Animal products, food of animal origin,... (Residues)	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat, milk and hen eggs	LC-MS/MS	Crook S.J. 2004 Ryan J. 2004b Benazeraf L. 2004 EU agreed
	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat 10 µg/l blood 5 µg/l Milk	LC-MS/MS	Tribolet, R. 2000 EU agreed
Soil, water, sediment,... (Environmental fate)	Primary	0.01 mg/kg	LC-MS/MS	Tummon O.K. 2004a EU agreed
	Primary	0.02 mg/kg loam, sandy loam, sand, overall soil	HPLC-UV	Kuhne-Thu H. 1997a Kuhne-Thu H. 1997b EU agreed

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

Component of residue definition: 1,2,4-triazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products,... (Residues)	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC-MS/MS	Longhi, D. 2021c (KCP 5.1.2-05)
	ILV (rapeseed)			Rigamonti E. 2022c (on-going) (KCP 5.1.2-06)
	ILV (rapeseed seeds)			Rigamonti E. 2022d (on-going) (KCP 5.1.2-07)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC-MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)
	ILV (dried apples)			Rigamonti E. 2022e (on-going) (KCP 5.1.2-9)
	ILV (dried tomatoes)			Rigamonti E. 2022f (on-going) (KCP 5.1.2-10)
Soil, water, sediment,... (Environmental fate)	Primary	0.01 mg/kg overall soil	HPLC-UV	Formica G. 1992a Sack S. 1994 Kuhne-Thu H. 2000 EU agreed
	Primary	0.05 mg/kg overall soil	HPLC-UV	Formica G. 1992a Sack S. 1994 Kuhne-Thu H. 2000 EU agreed

Table 5.2-6: Validated methods for the generation of pre-authorization data

Component of residue definition: Triazole-alanine				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products,... (Residues)	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC-MS/MS	Longhi, D. 2021c (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and	HPLC-MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

Component of residue definition: Triazole-alanine				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
		processed commodities		

Table 5.2-7: Validated methods for the generation of pre-authorization data

Component of residue definition: Triazole-lactic acid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products,... (Residues)	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC-MS/MS	Longhi, D. 2021c (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC-MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

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

Component of residue definition: Triazole-acetic acid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products,... (Residues)	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC-MS/MS	Longhi, D. 2021c (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC-MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

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)

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	Reg. (EU) 2019/552
Plant, high oil content		0.5 mg/kg	Reg. (EU) 2019/552
Muscle	Difenoconazole	0.05 mg/kg	Reg. (EU) 2019/552
Milk		0.005* mg/kg	Reg. (EU) 2019/552
Eggs		0.05* mg/kg	Reg. (EU) 2019/552
Fat		0.05 mg/kg	Reg. (EU) 2019/552
Liver, kidney		0.2 mg/kg	Reg. (EU) 2019/552
Soil (Ecotoxicology)	Difenoconazole	0.16 mg/kg bw/day	AOEL for difenoconazole EFSA Journal 2011;9(1):1967
Drinking water (Human toxicology)	Difenoconazole	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Difenoconazole	0.0056 mg a.s./L	NOEC for difenoconazole EFSA Journal 2011;9(1):1967
Air	Difenoconazole	0.16 mg/kg bw/day	AOEL for difenoconazole EFSA Journal 2011;9(1):1967
Tissue (meat or liver)	Difenoconazole	Not required	Not classified as T / T+
Body fluids		Not required	Not classified as T / T+

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

An overview on the acceptable methods and possible data gaps for analysis of Difenoconazole in plant matrices is given in the following tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

zRMS coment (EFSA Journal 2021;19(2):6407):

Analytical methods for the determination of difenoconazole residues were assessed in the framework of the EU pesticides peer review (Sweden, 2006; EFSA, 2011a). They are based on liquid chromatography with tandem mass spectrometry (LC–MS/MS) and were validated in high water content commodities (apples, lettuces) at the LOQ of 0.02 mg/kg, in dry commodities (wheat grain) at the LOQ of 0.05 mg/kg and in high oil content commodities (rapeseed) at the LOQ of 0.05 mg/kg. A QuEChERS method as reported in the European Standard EN 15662:2008 (CEN, 2008) is also available for the analysis of difenoconazole residues in high water, acidic and dry/high starch content commodities with an LOQ of 0.01 mg/kg (EFSA, 2017, 2018a).

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/ High oil content	Primary ILV	0.02 mg/kg: Apple, lettuce, 0.05 mg/kg: wheat (grain), 0.05 mg/kg: oil seed rape	LC-MS/MS	Steinhauser S. 2004a (primary) Schulz H. 2004 (ILV) EU agreed
	Primary	0.05 mg/kg Tomatoes, potatoes	GC NPD	Williams W.L., Shoffner K.P. 1987 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
	Primary	0.05 mg/kg wheat raw agric.	GC NPD	Williams W.L. 1988 Whetzel J.E. 1990 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
	Primary	0.03 mg/kg Wheat raw agric., apple/pear, carrot	GC NPD	Ross J. 1991 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
	Primary	0.04 mg/kg carrot	GC ECD	Bussy L., Maffei M. 1993 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC MS/MS	Longhi, D. 2021a (KCP 5.1.2-01)

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
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC MS/MS	Longhi, D. 2021b (KCP 5.1.2-02)

Table 5.3 3: Validated methods for the generation of pre-authorization data

Component of residue definition: 1,2,4-triazole				
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/ High oil content	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC MS/MS	Longhi, D. 2021c (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

Table 5.3 4: Validated methods for the generation of pre-authorization data

Component of residue definition: Triazole-alanine				
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/ High oil content	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC MS/MS	Longhi, D. 2021e (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

Table 5.3-5: Validated methods for the generation of pre-authorization data

Component of residue definition: Triazole-lactic acid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content/ High oil content	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC MS/MS	Longhi, D. 2021e (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

Table 5.3-6: Validated methods for the generation of pre-authorization data

Component of residue definition: Triazole-acetic acid				
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/ High oil content	Primary and confirmatory	0.01 mg/kg wheat, barley, oilseed rape and processed commodities	HPLC MS/MS	Longhi, D. 2021e (KCP 5.1.2-05)
	Primary and confirmatory	0.01 mg/kg apple, carrot, tomato and processed commodities	HPLC MS/MS	Longhi, D. 2021d (KCP 5.1.2-08)

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

Table 5.3-7: Statement on extraction efficiency

	Method for products of plant origin
Not required, because: Required, available from:	Multiresidue methods are available for the extraction of this active substance. In compliance with SANCO/825/00 rev. 8.1, it is not necessary to address extraction efficiency since there aren't matrix groups for which residues are \geq LOQ. Longhi, D. 2021c (GLP-STUDY-21-108) Longhi, D. 2021a (GLP-STUDY-21-31) Longhi, D. 2021b (GLP-STUDY-21-32) Longhi, D. 2021d (GLP-STUDY-21-109)
Difenoconazole	
Available from:	14C metabolism studies on tomato (N-0964-0700) and potato (N-0964-0400) used acetonitrile/ water to efficiently extract radioactive residues (approximately

	Method for products of plant origin
	80 – 100% of the extractable residue). Please refer to DAR, Difenconazole Volume 3 B7.
No additional data required, because:	As the methods developed for the purpose of the present application involve an identical extraction system to that used in the QuEChERS multi-residue procedure, data on recoveries are sufficient to demonstrate the extraction system is adequate for extracting residues of difenoconazole from crop commodities.
TDMs	
EU data:	It is not possible to provide metabolism study based on TDMs as precursors (they are not an active ingredient) and since TDM are common metabolites of all the triazole-based active ingredients, information can be derived from the DAR of several triazole fungicides.
No additional data required, because:	- the method used was an official single residue monitoring method - the use of the selected solvent (acidic methanol, in the presence of water that is contained in the matrix or was added for the dried ones) was supported by methods already accepted for instance in the Prothioconazole or Difenconazole RAR.

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 Difenconazole in animal matrices is given in the following tables. No new/additional studies presented.

zRMS:

Due to the use of dichloromethane in the methods of Crook (2004), Ryan (2004) and Benazeraf (2004) new methods should be provided (post registration requirement in Poland – after renewal of active substance). Fulfillment of this requirement may be considered at the Member State level.

The Crook Ryan and Benazeraf methods are on the list of accepted methods in the DAR and in the Addendum and Corrigendum.

Table 5.3-84: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: Difenconazole				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	Rovetto, I, 2023 (1111.4F.SAG23)
	ILV	0.01 mg/kg	HPLC-MS/MS	Mattioli, B, 2023 (CH-0859-2023)
Bovine liver, kidney, muscle, fat, milk, blood and hen eggs	Primary ILV	0.01 mg/kg Bovine liver, kidney, muscle, fat, milk and hen eggs	GC-NPD	Crook S.J. 2004 (primary) Ryan J. 2004b Benazeraf L. 2004 (ILV) EU agreed

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
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	Rovetto, I, 2023 (1111.4F.SAG23)
	ILV	0.01 mg/kg	HPLC-MS/MS	Mattioli, B, 2023 (CH-0859-2023)
	Primary	0.01 mg/kg Milk 0.05 mg/kg Bovine liver, kidney, muscle, fat and hen eggs	LC MS/MS	Wurx R.E.M. 1994 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat 10 µg/l blood 5 µg/l Milk	LC MS/MS	Tribolet, R. 2000 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)
Component of residue definition: Triazole-derivate metabolites (TDM)				
Honey	Primary	0.01 mg/kg	HPLC-MS/MS	Rovetto, I, 2023 (1111.4F.SAG23)
	ILV	0.01 mg/kg	HPLC-MS/MS	Mattioli, B, 2023 (0859-2023)

Table 5.3-5: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: Metabolite CGA 205375				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Bovine liver, kidney, muscle, fat, milk, blood and hen eggs	Primary ILV	0.01 mg/kg Bovine liver, kidney, muscle, fat, milk and hen eggs	LC-MS/MS	Crook S.J. 2004 (primary) Ryan J. 2004b Benazeraf L. 2004 (ILV) EU agreed
	Primary	0.01 mg/kg Bovine liver, kidney, muscle, fat 10 µg/l blood 5 µg/l Milk	LC-MS/MS	Tribolet, R. 2000 EU agreed not EU agreed (corrigendum/addendum Vol. 3, B.5, 2020)

Table 5.3-6: Statement on extraction efficiency

	Method for products of animal origin
Not required, because:	Multiresidue methods are available for the extraction of this

	Method for products of animal origin
	active substance. In compliance with SANCO/825/00 rev. 8.1, it is not necessary to address extraction efficiency since there aren't matrix groups for which residues are \geq LOQ.

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 5.3-7: Validated methods for soil

Component of residue definition: Difenoconazole			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg	LC-MS/MS	Tummon O.K. 2004a EU agreed
Primary	0.04-0.05 mg/kg silty clay loam, everglades peat, silt loam, sand, clay, sandy loam	GC-NPD GC-ECD	Williams, R.K. 1986 Kuhne-Thu H. 1990b Kuhne-Thu H. 1990a EU agreed
Primary	0.04 mg/kg overall soil	GC-ECD	Williams, R.K and Shoffner, K.P. 1987 Kuhne-Thu H. 2000 Kuhne-Thu H. 1990c Kuhne-Thu H. 1990d Kuhne-Thu H. 1991a Kuhne-Thu H. 1991b Kuhne-Thu H. 1990b Kuhne-Thu H. 1990a EU agreed
Primary	0.02 mg/kg sandy loam and loam	GC-ECD	Ross, J. 1991 Kuhne-Thu H. 2000 Kuhne-Thu H. 1992a EU agreed
Primary	0.05 mg/kg overall soil	GC-ECD	Kuhne-Thu H. 1996 Tack T. 1995 EU agreed
Primary and confirmatory	42.3 µg/kg soil	HPLC-MS triple quadrupole detector	Garagna, D. 2021a (KCP 5.1.2-11)

Table 5.3-8: Validated methods for soil

Component of residue definition: 1,2,4-triazole			
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.K. 2004a

Component of residue definition: 1,2,4-triazole			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
			EU agreed
Primary	0.02 mg/kg loam, sandy loam, sand, overall soil	HPLC-UV	Kuhne-Thu H. 1997a Kuhne-Thu H. 1997b EU agreed

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

zRMS comment:

Methods Garagna 2021 a-c, as indicated in table 5.2-3 are intended as pre-submission requirements

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.

zRMS comment:

An independent laboratory validation (ILV) of the analytical method for difenoconazole in drinking water is required according to Regulation (EC) No 283/2013. This is a data gap (post registration requirement). The methods of Garagna, 2021 are not acceptable for monitoring, because they were not validated for surface water and the LOQs are not sufficiently low to enforce the drinking water limit. Methods Garagna 2021 a-c, as indicated in table 5.2-3 are intended as pre-submission requirements

Table 5.3-9: Validated methods for water

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 and Surface water	Primary	0.05-0.1 µg/l surface water and drinking water (potable)	GC-ECD	Tribolet, R. 1999a Tribolet, R. 1999b EU agreed
	Confirmatory	0.05-0.1 µg/l surface water and drinking water (potable)	HPLC-UV	Tribolet, R. 1999a Tribolet, R. 1999b EU agreed
	Primary	0.1 µg/l HPLC-grade and drinking water (potable)	GC-ECD	Tribolet, R. 1990 EU agreed
	Confirmatory	0.1 µg/l HPLC-grade and drinking water (potable)	HPLC-UV	Tribolet, R. 1990 EU agreed

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
Stock Solutions	Primary and confirmatory	0.23 mg/L Stock Solutions	HPLC-MS triple quadrupole detector	Garagna, D. 2021b (KCP 5.1.2-12)
Aqueous samples	Primary and confirmatory	9.5 µg/L – 9.6 µg/L aqueous samples	HPLC-MS triple quadrupole detector	Garagna, D. 2021c (KCP 5.1.2-13)

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

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

An overview on the acceptable methods and possible data gaps for analysis of Difenoconazole in air is given in the following tables. No new/ additional studies presented.

Table 5.3-10: Validated methods for air

Component of residue definition: Difenoconazole			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.99 ng/l	LC-MS/MS	Tummon O.J. 2004b EU agreed
Primary	0.1 µg/l	GC-ECD	Tribolet, R. 1992 Tribolet, R. 1996 EU agreed

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

Difenoconazole is not classified as toxic or highly toxic, therefore no analytical method is required.

zRMS comment: An analytical method for difenoconazole in body fluids is required according to Regulation (EC) No 283/2013. This is a data gap (post registration requirement)

5.3.2.8 Other studies/ information

Not necessary.

Appendix 1 Lists of data considered in support of the evaluation

MS to blacken authors of vertebrate studies in the version made available to third parties/public.

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1/01	Urbani, M.	2021a	Difenoconazole 250 g/L EC greener – IN005B1570: Validation of the Analytical Method for the Determination of the Active Ingredient Content Report No CH – 0330/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.1/02	Urbani, M.	2021b	Difenoconazole 250 g/L EC greener – IN005B1570: Validation of the Analytical Method for the Determination of Toluene as Relevant Impurity Content Report No CH – 0331/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/01	Longhi, D.	2021a	Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities Report No GLP-STUDY-21-31 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/02	Longhi, D.	2021b	Validation of an analytical method for the quantification of Difenoconazole in apple, carrot, tomato and processed commodities Report No GLP-STUDY-21-32 LabAnalysis s.r.l.	N	INDOFIL Industries (Netherlands) B.V.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Unpublished		
KCP 5.1.2/03	Rigamonti, E.	2022a	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole in Dried apples Report No 1079/2021 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/04	Rigamonti, E.	2022b	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole in Dried tomatoes Report No 1080/2021 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/05	Longhi, D.	2021c	Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities Report No GLP-STUDY-21-108 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/06	Rigamonti, E.	2022c	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed) Report No GLP-STUDY-1085/2021 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/07	Rigamonti, E.	2022d	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Rapeseed seeds Report No GLP-STUDY-1090/2021	N	INDOFIL Industries (Netherlands)

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
			LabAnalysis s.r.l. GLP Unpublished		B.V.
KCP 5.1.2/08	Longhi, D.	2021d	Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in apple, carrot, tomato and processed commodities Report No GLP-STUDY-21-109 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/09	Rigamonti, E.	2022e	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Dried apples Report No GLP-STUDY-1088/2021 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/10	Rigamonti, E.	2022f	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Dried tomatoes Report No GLP-STUDY-1089/2021 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/11	Garagna, D.	2021a	Validation of the Analytical Method for the Determination of Difenconazole residues in soil samples of Difenconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests Report No. 0368/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.

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 5.1.2/12	Garagna, D.	2021b	Validation of the Analytical Method for the Determination of Difenoconazole content in Stock Solutions of Difenoconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests Garagna, D. 2021 Report No. 0782/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/13	Garagna, D.	2021c	Validation of the Analytical Method for the Determination of Difenoconazole residues in aqueous samples of Difenoconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests Report No. 0367/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/14	Nichetti, S.	2022a	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed) Report No CH-1084/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/15	Nichetti, S.	2022b	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Rapeseed seeds Report No CH-1083/2021 ChemService S.r.l. Controlli e Ricerche GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.1.2/16	Tediosi, E	2023c	Difenoconazole 250 g/L EC greener-IN005B1570: Validation of the Analytical Method for the Determination of Difenoconazole content in Feeding Solutions and in Aqueous Stock Solutions for Honey Bees tests Report No CH-0102-2023	N	INDOFIL Industries (Netherlands) B.V.

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
			ChemService S.r.l. Controlli e Ricerche GLP Unpublished		
KCP 5.2	Longhi D.	2022	IN005B1570: equipment cleaning procedure Study No. GLP-STUDY-LBN-0040-2022 LabAnalysis S.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.2/01	Rovetto, I.	2023	Magnitude of the residue of difenoconazole, prothioconazole, prothioconazole-desthio and triazole-derivative metabolites (TDMs) in honey after one application of IN233C1560 380 EC on Phacelia crop under semi field conditions in four trials in Northern Europe and Southern Europe – 2023 Study code: 1111.4F.SAG23 GLP Not Published	N	INDOFIL Industries (Netherlands) B.V.
KCP 5.2/02	Mattioli, B.	2023	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivative Metabolites (TDMs) residue in Honey Study No. CH – 0859-2023 GLP Not Published	N	INDOFIL Industries (Netherlands) B.V.
	Longhi, D.	2023	Validation of an analytical method for the quantification of Difenconazole, Prothioconazole and Prothioconazole-desthio in honey. Report No LBN-0092-2023 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.

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
	Longhi, D.	2023	Study Plan amendment No.1 Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey. Report No LBN-0092-2023	N	INDOFIL Industries (Netherlands) B.V.
	Longhi, D.	2023	Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey. Report No LBN-0093-2023 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
	Longhi, D.	2023	Study Plan amendment No.1 Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey. Report No LBN-0093-2023 LabAnalysis s.r.l. GLP Unpublished	N	INDOFIL Industries (Netherlands) B.V.
	Mattioli, B.	2024	Statement	N	INDOFIL Industries (Netherlands) B.V.

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
			Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivative Metabolites (TDMs) residue in Honey Study No. CH – 0859-2023 Lab Analysis Life Science s.r.l.		

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 XX	Author	YYYY	Title Company Report N Source GLP/non GLP/GEP/non GEP Published/Unpublished	Y/N	Owner

The following tables are to be completed by MS

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for Difenoconazole

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

For the active ingredient and relevant impurity please refer to the studies described in the points 5.2.1.1 and 5.2.1.2 (KCP 5.1.1/01 Urbani, M., 2021a and KCP 5.1.1/02 Urbani, M. 2021b).

New residue studies have been conducted and are submitted and summarised in this section.

Furthermore, three new analytical methods have been validated in the context of ecotoxicological studies and are hereunder presented in support of this new dossier. A summary of these studies is presented in this Appendix.

A 2.1.1 Methods for post-authorization control and monitoring purposes pre-authorization data (KCP 5.2)

NOTE: Information on extraction efficiency is provided in Appendix 3

zRMS: statement provided by the applicant is accepted

For difenoconazole, as the methods developed for the purpose of the present application involve an identical extraction system to that used in the QuEChERS multi-residue procedure, data on recoveries are sufficient to demonstrate the extraction system is adequate for extracting residues of difenoconazole from crop commodities.

For TDMs, the method used was an official single residue monitoring method the use of the selected solvent (acidic methanol, in the presence of water that is contained in the matrix or was added for the dried ones) was supported by methods already accepted in Difenoconazole RAR.

A 2.1.1.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

A 2.1.1.1.1 Analytical method 1 (Wheat, barley, oilseed rape and processed commodities)

A 2.1.1.1.1.1 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/01

Report Difenoconazole 250 g/L EC Greener – IN005B1570: Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desethio in wheat, barley, oilseed rape and processed commodities
Longhi, D. 2021

Study No. GLP-STUDY-21-31

Guideline(s):	Yes. <ul style="list-style-type: none">- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).- European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.- OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17.- “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for the determination of Difenoconazole and Prothioconazole-desthio in the tested matrices (AM-GLP-STUDY-21-31) was based on the QuEChERS method (EN 15662_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry).

Description of the method

Sample extraction

Plant matrices and processed commodities (whole plant, rapeseed seeds, wheat grain, white bread, straw): Aliquots of 5 g of specimen (2.5 for straw) were taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of the following amounts of LC-MS grade water:

Matrix	Water added (mL)
Whole plant(Rapeseed)	0
Rapeseed seeds	10
Grain (wheat)	10
Straw (wheat)	10
White bread (wheat)	10

Then, 20 mL of acetonitrile were added and the obtained mixture was vigorously shaken for one minute. After that, a packet of QuEChERS extraction salt (4.0 g MgSO₄, 1.0 g NaCl, 1.0 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation at 4500 rpm for 5 minutes. An aliquot of about 1 mL the organic supernatant was taken, transferred in a 2 mL HPLC glass vial and analysed with a HPLC-MS/MS system.

Beer:

Beer was analysed after a 5-fold dilution in a mixture of water/methanol 50:50 (about 5 g to 25 mL) and directly analysed with a HPLC-MS/MS system.

Rapeseed oil:

An aliquot of about 5 g of rapeseed oil was put in a 50 mL screw capped centrifuge PE test tube. Then, 15 mL of hexane were added, followed by 20 mL of a mixture of acetonitrile/water 80:20. The mixture was vigorously shaken for about one minute and then centrifuged at 4500 rpm for 5 minutes, in order to obtain 2 phases. An aliquot of the lower organic phase (acetonitrile) was taken and transferred to a 2 mL glass HPLC vial for final determination with a HPLC-MS/MS system.

Reference solutions preparation:

The following reference solutions were prepared:

Solution	Starting material	Weight/Volume	Final volume (mL) (acetonitrile)	Actual concentration (mg/L)
Difenoconazole stock solution (Difenoconazole ~ 2000 mg/L)	Difenoconazole reference material (purity: 95.4%)	20.56 mg	10	1961
Prothioconazole-desethio stock solution (Prothioconazole-desethio ~ 1000 mg/L)	Prothioconazole-desethio reference material (purity: 99.55%)	10.81 mg	10	1076
Solution A (~ 10 mg/L)	Difenoconazole stock solution + Prothioconazole-desethio stock solution	51 µL + 93 µL	10	10.0
Solution B (~ 1 mg/L)	Difenoconazole stock solution + Prothioconazole-desethio stock solution	5.1 µL + 9.3 µL	10	1.00

Matrix-matched standard solutions for plant matrices, processed commodities, rapeseed oil were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of Solution B	Final volume (mL)	Nominal concentration (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	2	0.5	0.002
L2	2.5	1	2.5	0.010
L3	5	1	5	0.020
L4	25	1	25	0.100
L5	50	1	50	0.200

1: calculated considering the nominal sample preparation (5.0 g to a final volume of 20.0 mL)

Matrix-matched analytical standard solutions for straw were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of Solution B	Final volume (mL)	Nominal concentration (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1.2	2	0.3	0.0024
L2	1	1	2.5	0.008
L3	2.5	1	5	0.020
L4	10	1	10	0.080
L5	25	1	25	0.200

1: calculated considering the nominal sample preparation (2.5 g to a final volume of 20.0 mL)

Matrix-matched analytical standard solutions for beer were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of Solution B	Final volume (mL)	Nominal concentration (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	2	0.5	0.0025
L2	2.5	1	2.5	0.0125
L3	5	1	5	0.025
L4	25	1	25	0.125
L5	50	1	50	0.250

1: calculated considering the nominal sample preparation (5.0 g to a final volume of 25.0 mL)

The analyses were carried out using a HPLC-MS/MS system according to the following conditions:

Instrument: HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470A

Column: Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm

Column temperature: 40°C

Flow: 0.6 mL/min

Injection volume: 2.5 µL

Mobile phase A: LC-MS grade water with 0.2 % formic acid and 5 mM ammonium formate

Mobile phase B: LC-MS grade methanol with 0.2 % formic acid and 5 mM ammonium formate

Elution: gradient of the following composition:

Time (min)	% A	% B
0	50	50
0.5	50	50
3	0	100

Stop time: 5 min

Post time: 1 min

Divert valve: 0 min. to waste, 1.5 min. to MS, 3 min. to waste

Source type: ESI

Gas temperature: 350°C

Gas flow (L/min): 5

Nebulizer (psi): 40

Sheath gas heater: 400°C

Sheath gas flow (L/min): 12

Capillary: positive mode 3500 V, negative mode 3000 V

Vcharging: 0

Acquiring mode: ESI positive and ESI negative, MRM (multi-reaction monitoring).

Calibration

The quantification of each analyte was made through the building of a calibration straight line with the external standard method. 5 matrix-matched analytical standard solutions were analysed in single injections

in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy) and Repeatability (Precision)

Recovery and repeatability (as precision, % RSD) data will be reported for the following fortification levels:

- LOQ level (5 replicates): 0.01 mg/kg
- 10xLOQ level (5 replicates): 0.1 mg/kg - or alternatively at a spiking level higher than maximum residue level found on field specimens.

Specificity (Selectivity)

This parameter will be evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by matrix interferences.

Using a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: samples, fortified samples, reference solutions at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements.

Matrix effect

Assessment of matrix effects will be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix, for all sample matrix used in the study. In order to nullify matrix effect, calibration curves for all matrices analysed will be prepared using matrix matched analytical standards.

Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample. It is the lowest concentration at which an analyte produces an instrumental signal at least 3 times higher than background noise of the chromatogram. It should be not higher than 30% of LOQ value and it will be considered as the lowest point of the instrumental calibration.

Limit of quantification (LOQ)

Limit of quantitation (LOQ) is defined as the lowest validated level with sufficient recovery and precision. Target LOQ for Difenoconazole and Prothioconazole-desthio in the sample analysed will be set to 0.01 mg/kg.

Confirmation:

A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring an additional SRM transition. The following data will be provided for a product ion scan of a 1 mg/L solution of Difenoconazole + Prothioconazole-desthio (Solution B), to justify the selection of the MS/MS transitions used: calibration data as recorded for primary detection, recovery and precision data as recorded for primary detection (at least for the 5 replicates at LOQ level) the recovery and precision mean values calculated on confirmatory ion must fulfill the same acceptable range reported above for primary detection.

Results and discussions

Method validation data can be summarised in the tables below.

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	Difenoconazole	0.01 (LOQ)	87.3	2.7	3.1	
		0.1 (10xLOQ)	87.4	5.7	6.5	
Rapeseed seeds	Difenoconazole	0.01 (LOQ)	85.1	1.9	2.3	
		0.1 (10xLOQ)	86.4	5.4	6.3	
Wheat grain	Difenoconazole	0.01 (LOQ)	88.9	2.1	2.4	
		0.1 (10xLOQ)	91.2	3.9	4.3	
Straw (wheat)	Difenoconazole	0.01 (LOQ)	91.4	8.6	9.4	
		0.1 (10xLOQ)	93.8	7.1	7.6	
Rapeseed oil	Difenoconazole	0.01 (LOQ)	88.2	3.8	4.3	
		0.1 (10xLOQ)	87.9	1.7	2.0	
White bread (wheat)	Difenoconazole	0.01 (LOQ)	100.1	7.7	7.7	
		0.1 (10xLOQ)	95.5	0.54	0.54	
Beer (barley)	Difenoconazole	0.01 (LOQ)	89.9	6.1	6.7	
		0.1 (10xLOQ)	84.9	1.5	1.8	

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	Prothioconazole-desthio	0.01 (LOQ)	85.4	7.2	8.5	
		0.1 (10xLOQ)	82.7	6.7	8.1	
Rapeseed seeds	Prothioconazole-desthio	0.01 (LOQ)	89.3	5.1	5.7	
		0.1 (10xLOQ)	81.5	5.3	6.5	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Wheat grain	Prothioconazole-desthio	0.01 (LOQ)	87.4	3.9	4.5	
		0.1 (10xLOQ)	86.7	5.6	6.4	
Straw (wheat)	Prothioconazole-desthio	0.01 (LOQ)	80.1	2.9	3.6	
		0.1 (10xLOQ)	86.9	5.4	6.3	
Rapeseed oil	Prothioconazole-desthio	0.01 (LOQ)	79.5	5.3	6.7	
		0.1 (10xLOQ)	77.9	1.1	1.4	
White bread (wheat)	Prothioconazole-desthio	0.01 (LOQ)	97.7	1.7	1.8	
		0.1 (10xLOQ)	96.0	0.41	0.42	
Beer (barley)	Prothioconazole-desthio	0.01 (LOQ)	87.3	9.5	10.8	
		0.1 (10xLOQ)	80.4	1.9	2.3	

Table A 3: Characteristics for the analytical method used for validation of Difenoconazole and Prothioconazole-desthio residues in wheat, barley, oilseed rape and processed commodities

	Difenoconazole	Prothioconazole-desthio
Specificity	MS spectrum provided: Yes Positive polarity m/z 406.2 – 251.1 m/z 406.2 – 188.4	MS spectrum provided: Yes Negative polarity m/z 312.2 – 69.8 m/z 312.2 – 125.0
Calibration (type, number of data points)	Rapeseed seeds : n = 5 y = 2133.411025x + 406.088099 R ² = 0.999	Rapeseed seeds : n = 5 y = 1033.676304x + -7.425110 R ² = 0.999
	Whole Plant (Rapeseed) : n = 5 y = 2393.089220 x + 525.166810 R ² = 0.999	Whole Plant (Rapeseed) : n = 5 y = 1240.335143 x + 41.225438 R ² = 0.999
	Grain (wheat) : n = 5 y = 2417.915699 x + 657.442734 R ² = 0.999	Grain (wheat) : n = 5 y = 1086.126498 x + 28.886023 R ² = 0.999
	Straw (wheat) : n = 5 y = 3702.782310 x +	Straw (wheat) : n = 5 y = 1318.462397 x +

	1318.496403 $R^2 = 0.999$	97.326629 $R^2 = 0.999$
	Rapeseed oil : n = 5 $y = 2778.122779 x + 355.419442$ $R^2 = 0.999$	Rapeseed oil : n = 5 $y = 1151.412873 x + 126.288342$ $R^2 = 0.999$
	White bread (wheat) : n = 5 $y = 2506.307430 x + 2064.880900$ $R^2 = 0.999$	White bread (wheat) : n = 5 $y = 1242.277359 x + 138.386250$ $R^2 = 0.999$
	Beer (barley) : n = 5 $y = 2736.071421 x + 1929.069427$ $R^2 = 0.999$	Beer (barley) : n = 5 $y = 1096.261065 x + 153.749773$ $R^2 = 0.999$
Calibration range	Range: 0.5 – 50.0 µg/L (0.00200 – 0.200 mg/kg) Beer range: 0.5 – 50.0 µg/L (0.00250 – 0.250 mg/kg) Straw (wheat) range: 0.3 – 25.0 µg/L (0.00240 – 0.200 mg/kg)	Range: 0.5 – 50.0 µg/L (0.00200 – 0.200 mg/kg) Beer range: 0.5 – 50.0 µg/L (0.00250 – 0.250 mg/kg) Straw (wheat) range: 0.3 – 25.0 µg/L (0.00240 – 0.200 mg/kg)
Assessment of matrix effects is presented	Matrix effects: Yes For wheat straw the matrix effect is considered significant, since it was found out of the acceptability ranges.	Matrix effects: Yes For wheat straw the matrix effect is considered significant, since it was found out of the acceptability ranges.
Limit of determination/quantification	LOD: 0.50 µg/L LOD for wheat: 0.3 µg/L LOQ: 0.01 mg/Kg	LOD: 0.50 µg/L LOD for wheat: 0.3 µg/L LOQ: 0.01 mg/Kg

Conclusion

A mean recovery of 60-120% with a Relative Standard Deviation $\leq 30\%$ was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.1.2 ILV Method for rapeseed

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference:	KCP 5.1.2/14
Report	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in whole plant (Rapeseed) Nichetti, S. 2022 Study No. GLP-STUDY-1084/2021
Guidelines:	Yes. - European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-31 (KCP 5.2.1-01) for the determination of difenoconazole and prothioconazole-desthio in in whole plant (Rapeseed) in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 10 mL of water and 20.0 mL of acetonitrile were added and the obtained mixture was vigorously shaken for 1 minute. After that a packet of QuEChERS extraction was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using fortified Whole Plant (Rapeseed) samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration.

The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ.

No interferences above the LOD were found in the control Whole Plant (Rapeseed) samples, the analysis of fortified samples at low and high level gave the following results.

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
Difenoconazole	251.1	Low	0.010	5	0.009	97.0	6.31	0.0
		High	0.097	5	0.093	95.9	4.16	0.0
Difenoconazole	188.4	Low	0.010	5	0.008	88.0	4.71	0.0
		High	0.097	5	0.094	97.2	6.70	0.0
Prothioconazole-desthio	69.8	Low	0.011	5	0.011	105.2	5.58	0.0
		High	0.110	5	0.109	100.3	0.95	0.0
Prothioconazole-desthio	125.0	Low	0.011	5	0.012	107.8	8.37	0.0
		High	0.110	5	0.110	101.0	1.17	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)	Spike High or 10 x LOQ (n = 5)	Overall (n=10)
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		Mean	RSD%	Mean	RSD%	Mean	RSD%
Difenoconazole	251.1	97.0	6.31	95.91	4.16	96.4	5.09
Difenoconazole	188.4	88.0	4.71	97.2	6.70	92.6	7.63
Prothioconazole-desthio	69.8	105.2	5.58	100.3	0.95	102.8	4.62
	125.0	125.0	125.0	125.0	125.0	125.0	125.0

According the SANTE/2020/12830 rev. 1 guideline's requirement, the mean recovery values must be in the range 70 to 120 %, with an RSD% lower than 20%.

Since all recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Whole Plant (Rapeseed) samples with an established L.O.Q of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC/MS/MS instrument with quantification by external standard, was shown to be specific for Difenoconazole and Prothioconazole-desthio residues in Whole Plant (Rapeseed).

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
Difenoconazole	406.2	Quantifier	251.1	-5
Prothioconazole-desthio	312.2	Quantifier	69.8	-17

Not significant matrix effects for Difenoconazole and Prothioconazole-desthio residues in Whole Plant (Rapeseed) matrix were found ($< \pm 20\%$). Therefore, the calibration standards could be prepared in solvent or in matrix. A matrix-matched calibration standards were used throughout the entire study.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ. The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for each analyte, corresponding to about 0.002 mg/kg in Whole Plant (Rapeseed) (30% of the LOQ). The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (70 – 120% with % RSD < 20%), was a final injected solution of about 2.50 µg/L for each analyte, corresponding to about 0.010 mg/kg in Whole Plant (Rapeseed).

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
Difenoconazole	0.50	0.002	2.50	0.010
Prothioconazole-desthio	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Analyte results calculated as < 0.002 mg/kg (LOD) are classified as not detectable (n.d.).

Analyte results calculated as greater than the limit of detection but less than the limit of quantification, are designated as < 0.010 mg/kg.

If the analyte content is calculated as greater than 0.200 mg/kg in Whole Plant (Rapeseed), the final solution must be suitably diluted using volumetric glassware to fit in the calibration range.

Confirmation

Since the analysis by HPLC using external standards and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
Difenoconazole	quantifier	406.2	251.1
	qualifier		188.4
Prothioconazole-desthio	quantifier	312.2	69.8
	qualifier		125.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in rapeseed with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.3 ILV Method for rapeseed seeds

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/15

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in rapeseed seeds
Nichetti, S. 2022
Study No. GLP-STUDY-1083/2021

Guidelines: Yes.
- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-31 (KCP 5.2.1-01) for the determination of difenoconazole and prothioconazole-desthio in in whole plant (Rapeseed seeds) in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 10 mL of water and 20.0 mL of acetonitrile were added and the obtained mixture was vigorously shaken for 1 minute. After that a packet of QuEChERS extraction was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using fortified Rapeseed seeds samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration. The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ. No interferences above the LOD were found in the control Rapeseed seeds samples, the analysis of fortified samples at low and high level gave the following results.

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
Difenoconazole	251.1	Low	0.010	5	0.010	105.3	6.28	0.0
		High	0.098	5	0.106	109.7	1.23	0.0
Difenoconazole	188.4	Low	0.010	5	0.009	90.9	13.53	0.0
		High	0.098	5	0.106	109.0	4.02	0.0
Prothioconazole-desthio	69.8	Low	0.011	5	0.010	89.4	16.04	0.0
		High	0.111	5	0.108	97.9	1.46	0.0
Prothioconazole-desthio	125.0	Low	0.011	5	0.011	98.2	16.33	0.0
		High	0.111	5	0.109	98.7	2.53	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
Difenoconazole	251.1	105.3	6.28	109.7	1.23	107.5	4.71
Difenoconazole	188.4	90.9	13.53	109.0	4.02	100.0	12.92
Prothioconazole-desthio	69.8	89.4	16.04	97.9	1.46	93.6	11.32
Prothioconazole-desthio	125.0	98.2	16.33	98.7	2.53	98.5	10.99

According the SANTE/2020/12830 rev. 1 guideline's requirement, the mean recovery values must be in the range 70 to 120 %, with an RSD% lower than 20%.

Since all recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Rapeseed seeds samples with an established LOQ of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC/MS/MS instrument with quantification by external standard, was shown to be specific for Difenoconazole and Prothioconazole-desthio residues in Rapeseed seeds samples.

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
Difenoconazole	406.2	Quantifier	251.1	-5
Prothioconazole-desthio	312.2	Quantifier	69.8	-17

Not significant matrix effects for Difenoconazole and Prothioconazole-desthio residues in Rapeseed seed matrix were found ($< \pm 20\%$). Therefore, the calibration standards could be prepared in solvent or in matrix. A matrix-matched calibration standards were used throughout the entire study.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ.

The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for each analyte, corresponding to about 0.002 mg/kg in Rapeseed seeds (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (70 – 120% with % RSD < 20%), was a final injected solution of about 2.50 µg/L for each analyte, corresponding to about 0.010 mg/kg in Rapeseed seeds

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
Difenoconazole	0.50	0.002	2.50	0.010
Prothioconazole-desthio	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Confirmation

Since the analysis by HPLC using external standards and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
Difenoconazole	quantifier	406.2	251.1
	qualifier		188.4
Prothioconazole-desthio	quantifier	312.2	69.8
	qualifier		125.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in rapeseed seeds with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.2 Analytical method 2

A 2.1.1.1.2.1 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Report	Difenoconazole 250 g/L EC Greener – IN005B1570: Validation of an analytical method for the quantification of Difenoconazole in apple, carrot, tomato and processed commodities Longhi, D. 2021 Study No. GLP-STUDY-21-32
Guideline(s):	Yes. <ul style="list-style-type: none">- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).- European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.- OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17.- “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method”- GLP-STUDY-21-31, “Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desethio in wheat, barley, oilseed rape and processed commodities”, Test Facility: LabAnalysis srl, Study Director: Diego Longhi
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for the determination of Difenoconazole in the tested matrices (AM1-GLP-STUDY-21-32) was based on the QuEChERS method (EN 15662_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry).

Description of the method

Sample extraction

Plant matrices and processed (apple, tomato, carrot, dried apple, dried tomato):

Aliquots of 5 g of specimen were taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of the following amounts of LC-MS grade water:

Matrix	Water added (mL)
Apple	0
Tomato	0
Carrot	10*
Dried apple	8.5
Dried tomato	8.5

* The addition of water in carrot was necessary since preliminary tests showed poor recoveries if water was not added

Then, 20 mL of acetonitrile were added and the obtained mixture was vigorously shaken for one minute. After that, a packet of QuEChERS extraction salt (4.0 g MgSO₄, 1.0 g NaCl, 1.0 g trisodium citrate dehy-

drate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation at 4500 rpm for 5 minutes. An aliquot of about 1 mL the organic supernatant was taken, transferred in a 2 mL HPLC glass vial and analysed with a HPLC-MS/MS system.

Apple juice:

Apple juice was analysed after a 5-fold dilution in a mixture of water/methanol 50:50 (about 5 g to 25 mL) and directly analysed with a HPLC-MS/MS system.

Reference solutions preparation

The following reference solutions were prepared:

Solution	Starting material	Weight/Volume	Final volume (mL) (acetonitrile)	Actual concentration (mg/L)
Difenoconazole stock solution (Difenoconazole ~ 2000 mg/L)	Difenoconazole reference material (purity: 95.4%)	20.56 mg	10	1961
Solution A (~ 10 mg/L)	Difenoconazole stock solution	51 µL	10	10.0
Solution B (~ 1 mg/L)	Difenoconazole stock solution	5.1 µL	10	1.00

Matrix-matched analytical standard solutions for plant matrices and processed commodities were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of Solution B	Final volume (mL)	Nominal concentration (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	2	0.5	0.002
L2	2.5	1	2.5	0.010
L3	5	1	5	0.020
L4	25	1	25	0.100
L5	50	1	50	0.200

1: calculated considering the nominal sample preparation (5.0 g to a final volume of 20.0 mL)

Matrix-matched analytical standard solutions for apple juice were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of Solution B	Final volume (mL)	Nominal concentration (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	2	0.5	0.0025
L2	2.5	1	2.5	0.0125
L3	5	1	5	0.025
L4	25	1	25	0.125
L5	50	1	50	0.250

1: calculated considering the nominal sample preparation (5.0 g to a final volume of 25.0 mL)

The analyses were carried out using a HPLC-MS/MS system according to the following conditions:

Instrument: HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470A

Column: Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm
Column temperature: 40°C
Flow: 0.6 mL/min
Injection volume: 2.5 µL
Mobile phase A: LC-MS grade water with 0.2 % formic acid and 5 mM ammonium formate
Mobile phase B: LC-MS grade methanol with 0.2 % formic acid and 5 mM ammonium formate
Elution: gradient of the following composition:

Time (min)	% A	% B
0	50	50
0.5	50	50
3	0	100

Divert valve: 0 min. to waste, 1.5 min. to MS, 3 min. to waste
Source type: ESI
Gas temperature: 350°C
Gas flow (L/min): 5
Nebulizer (psi): 40
Sheath gas heater: 400°C
Sheath gas flow (L/min): 12
Capillary: positive mode 3500 V, negative mode 3000 V
Vcharging: 0
Acquiring mode: ESI positive, MRM (multi-reaction monitoring).

Calibration

The quantification of each analyte was made through the building of a calibration straight line with the external standard method. 5 matrix-matched analytical standard solutions were analysed in single injections in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy) and Repeatability (Precision)

Recovery and repeatability (as precision, % RSD) data will be reported for the following fortification levels:

- LOQ level (5 replicates): 0.01 mg/kg
- 10xLOQ level (5 replicates): 0.1 mg/kg - or alternatively at a spiking level higher than maximum residue level found on field specimens.

Specificity (Selectivity)

This parameter will be evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by matrix interferences.

Using a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: samples, fortified samples, reference solutions at the LOQ level in order to assess the presence or absence of interfering signals. No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements.

Matrix effect

Assessment of matrix effects will be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix, for all sample matrix used in the study. In order to nullify matrix effect, calibration curves for all matrices analysed will be prepared using matrix matched analytical standards.

Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample. It is the lowest concentration at which an analyte produces an instrumental signal at least 3 times higher than background noise of the chromatogram. It should be not higher than 30% of LOQ value and it will be considered as the lowest point of the instrumental calibration.

Limit of quantification (LOQ)

Limit of quantitation (LOQ) is defined as the lowest validated level with sufficient recovery and precision. Target LOQ for Difenoconazole in the sample analysed will be set to 0.01 mg/kg.

Confirmation:

A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring an additional SRM transition. The following data will be provided for the additional ion: calibration data as recorded for primary detection, recovery and precision data as recorded for primary detection (at least for the 5 replicates at LOQ level) the recovery and precision mean values calculated on confirmatory ion must fulfill the same acceptable range reported above for primary detection.

Results and discussions

Method validation data can be summarised in the tables below.

Table A 4: Recovery results from method validation of Difenoconazole using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Apple	Difenoconazole	0.01 (LOQ)	95.1	2.4	2.5	
		0.1 (10xLOQ)	90.6	3.5	3.9	
Tomato	Difenoconazole	0.01 (LOQ)	88.6	2.3	2.6	
		0.1 (10xLOQ)	89.7	1.6	1.8	
Carrot	Difenoconazole	0.01 (LOQ)	91.8	1.1	1.1	
		0.1 (10xLOQ)	103.1	3.3	3.2	
Dried apple	Difenoconazole	0.01 (LOQ)	96.8	5.4	5.6	
		0.1 (10xLOQ)	98.3	4.2	4.2	
Apple juice	Difenoconazole	0.01 (LOQ)	97.8	4.8	4.9	
		0.1 (10xLOQ)	102.0	1.5	1.5	
Dried tomato	Difenoconazole	0.01 (LOQ)	104.3	3.9	3.8	
		0.1 (10xLOQ)	108	2.7	2.5	

Table A 5: Characteristics for the analytical method used for validation of Difenoconazole residues in apple, carrot, tomato and processed commodities

	Difenoconazole
Specificity	<p>MS spectrum provided: Yes m/z 406.2 – 251.1 (primary) m/z 406.2 – 188.4 (confirmatory)</p> <p>Difenoconazole signals resulted lower than the instrumental limit of detection (LOD) for both primary and confirmatory detection, therefore the selectivity of the analytical method resulted proven.</p>
Calibration (type, number of data points)	<p>n = 5 Apple: $y = 1936.486257 + 1708.868073x$ $R^2 = 0.999$</p> <p>n = 5 Carrot: $y = 2588.634721x + 2011.755585$ $R^2 = 0.999$</p> <p>n = 5 Tomato: $y = 1635.518915x + 1469.715008$ $R^2 = 0.999$</p> <p>n = 5 Apple juice : $y = 3161.571008x + 1714.490169$ $R^2 = 0.999$</p> <p>n = 5 Dried apple : $y = 2855.669088x + 5730.950925$ $R^2 = 0.999$</p> <p>n = 5 Dried tomato: $y = 2899.614212x + 214.243402$ $R^2 = 0.999$</p>
Calibration range	<p>Range 0.5 µg/L – 50.0 µg/L (from 20% of LOQ to 100% above 10xLOQ) Apple juice range: 0.5 – 50.0 (from 25% of LOQ to 150% above 10xLOQ)</p> <p>Range: 0.00200 mg/kg – 0.200 mg/kg Apple juice range: 0.0025 mg/kg – 0.250 mg/kg</p>
Assessment of matrix effects is presented	<p>Yes</p> <p>Matrix effects, expressed in % enhancement or suppression of signal, were considered not significant according to the SANTE/2020/12830 rev.1 guideline, since the analyte responses in matrix matched calibration solutions are $< \pm 20\%$ of the analyte responses in the calibration solutions prepared in solvent (acetonitrile and water/methanol 50:50 v/v). Since the matrix effect is negligible, the calibration can also be done using standards prepared in solvent.</p>
Limit of determination/quantification	<p>LOD: 0.50 µg/L (corresponding to (0.002 mg/kg)) LOQ: 0.01 mg/kg</p>

Conclusion

A mean recovery of 60-120% with a Relative Standard Deviation $\leq 30\%$ was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.1.2.2 ILV Method for dried apples

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference:	KCP 5.1.2/03
Report	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole in Dried apples Rigamonti, E. 2022 Study No. GLP-STUDY-1079/2021
Guidelines:	Yes. - European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-32 (KCP 5.1.2-02) for the determination of difenoconazole in dried apples in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 8.5 mL of water and 20.0 mL of acetonitrile were added and the obtained mixture was vigorously shaken for 1 minute. After that a packet of QuEChERS extraction was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using Dried Apples samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration. The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ.

No interferences above the LOD were found in the control Dried Apples samples, the analysis of fortified samples at low and high level gave the following results.

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery	RSD%	Interference (%)
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						(%)		
Difenoconazole	251.1	Low	0.010	5	0.010	96.8	12.00	0.0
		High	0.100	5	0.103	103.9	2.58	0.0
Difenoconazole	188.4	Low	0.010	5	0.009	93.3	8.34	0.0
		High	0.100	5	0.103	103.6	3.27	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
Difenoconazole	251.1	96.8	12.00	103.9	2.58	100.4	8.76
Difenoconazole	188.4	93.3	8.34	103.6	3.27	98.5	7.96

Since all recovery values for the analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the correct range (70 to 120 %, with an RSD% lower than 20%), this criterion was fulfilled and therefore, the analytical method can be considered suitable to quantify Difenoconazole in Dried Apples samples

Specificity (Selectivity)

The analytical method, using the HPLC/MS/MS instrument with quantification by external standard, was shown to be specific for Difenoconazole residue in Dried Apples samples.

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
Difenoconazole	406.2	Quantifier	251.1	40

A significant matrix effects for Difenoconazole in the Dried Apples matrix was found ($> \pm 20\%$). Therefore, matrix-matched calibration standards were used throughout the entire study.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ. The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for the analyte, corresponding to about 0.002 mg/kg in Dried Apples (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (70 – 120% with % RSD < 20%), was a final injected solution of about 2.50 µg/L for the analyte, corresponding to about 0.010 mg/kg in Dried Apples.

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
Difenoconazole	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for the analyte and both mass transitions.

Analyte results calculated as < 0.002 mg/kg (LOD) are classified as not detectable (n.d.).

Analyte results calculated as greater than the limit of detection but less than the limit of quantification, are designated as < 0.010 mg/kg.

If the analyte content is calculated as greater than 0.200 mg/kg in Dried Apples, the final solution must be suitably diluted using volumetric glassware to fit in the calibration range.

Confirmation

Since the analysis by HPLC using an external standard and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode is highly specific and gave quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
Difenoconazole	quantifier	406.2	251.1
	qualifier		188.4

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in dried apples with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.2.3 ILV Method for dried tomatoes

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/04

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole in Dried tomatoes
 Rigamonti, E. 2022
 Study No. GLP-STUDY-1080/2021

Guidelines: Yes.
 - European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-32 (KCP 5.1.2-02) for the determination of difenoconazole in dried tomatoes in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots were taken from the homogenised frozen samples and put in a screw capped centrifuge PE test tube followed by the addition of LC-MS grade water. Then, acetonitrile was added and the obtained mixture was vigorously shaken. After that, a packet of QuEChERS extraction salt (4.0 g MgSO₄, 1.0 g NaCl, 1.0 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using dried tomatoes samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration. The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ.

No interferences above the LOD were found in the control dried tomatoes samples, the analysis of fortified samples at low and high level gave the following results.

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
Difenoconazole	251.1	Low	0.010	5	0.011	110.1	9.54	0.0
		High	0.100	5	0.079	81.3	14.21	0.0
Difenoconazole	188.4	Low	0.010	5	0.011	108.1	7.07	0.0
		High	0.100	5	0.081	82.6	13.14	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
Difenoconazole	251.1	110.1	9.54	81.3	14.21	95.7	19.24
Difenoconazole	188.4	108.1	7.07	82.6	13.14	95.4	16.90

According the SANTE/2020/12830 rev. 1 (2021) guideline's requirement, the mean recovery values must be in the range 70 to 120 %, with an RSD% lower than 20%.

Since all recovery values for the analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the correct range, this criterion was fulfilled and therefore, the analytical method can be considered suitable to quantify Difenoconazole in Dried Tomatoes samples.

Specificity (Selectivity)

The analytical method, using the HPLC/MS/MS instrument with quantification by external standard, was shown to be specific for difenoconazole residue in dried tomatoes samples.

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
Difenoconazole	406.2	Quantifier	251.1	-1

Not significant matrix effect for difenoconazole in the dried tomatoes matrix was found ($< \pm 20\%$).

Therefore, the calibration standards could be prepared in solvent or in matrix. Matrix-matched calibration standards were used throughout the entire study.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ.

The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for the analyte, corresponding to about 0.002 mg/kg in Dried Tomatoes (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (70 – 120% with % RSD < 20%), was a final injected solution of about 2.50 µg/L for the analyte, corresponding to about 0.010 mg/kg in dried tomatoes.

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
Difenoconazole	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for the analyte and both mass transitions. Analyte results calculated as < 0.002 mg/kg (LOD) are classified as not detectable (n.d.). Analyte results calculated as greater than the limit of detection but less than the limit of quantification, are designated as < 0.010 mg/kg.

If the analyte content is calculated as greater than 0.200 mg/kg in dried tomatoes, the final solution must be suitably diluted using volumetric glassware to fit in the calibration range.

Confirmation

Since the analysis by HPLC using an external standard and MS triple quadrupole detector (HPLC/MS/MS) in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary. The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
Difenoconazole	quantifier	406.2	251.1
	qualifier		188.4

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in dried tomatoes with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.3 Analytical method 3

A 2.1.1.1.3.1 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/05

Report Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities
Longhi, D. 2021

Study No. GLP-STUDY-21-108

Guideline(s):	Yes.
	<ul style="list-style-type: none"> - European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Postapproval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021). - European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods. - OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17. - “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPE-PO-Method)- Method 8 (M8)”
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The validation of the analytical method was carried out under GLP compliance to SANTE/2020/12830 Rev.1 guideline. The analytical determination was carried out using a HPLC-MS/MS method validated in the GLP study coded GLP-STUDY-21-108.

Description of the method

Sample extraction

An appropriate aliquot of each specimen was taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of 100 µL of the internal standard solution TDM ISTD MIX (2 mg/L of each internal standard) and by the following amounts of deionized water (added on the basis of QuPPE-PO-Method and considering the theoretical water content of each matrix):

Matrix	Matrix group	Sample weight (g)	Water added (mL)
Whole Plant (Rapeseed)	high water content commodity	5	5
Rapeseed seeds	high oil content commodity	5	10
Grain (wheat)	dry commodity	5	10
Straw (wheat)	dry commodity	2.5	10
Rapeseed oil	high oil content commodity	5	10
White bread (wheat)	dry commodity	5	10
Beer (barley)	high water content commodity	10	0

Then, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The volume of the final extract is considered to be 20 mL: little variation due to the actual water content of each sample are corrected by the presence of the internal standard, that is added to produce a concentration in the final extract nominally of 10 µg/L of each compound.

The separation of the liquid phase from the solid one was achieved by centrifugation at 5000 rpm for 5 minutes. An aliquot of about 1 mL the supernatant was taken, filtered with a 0.45 µm PVDF filter and transferred in a 2 mL HPLC glass vial for the final analysis with a HPLC-DMS-MS/MS system.

Reference solutions preparation:

The following reference solutions were prepared:

Stock solution	Preparation date (dd/mm/yyyy)	Starting material	Weight (mg)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
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1,2,4-TRZ stock solution	25/08/2021	1,2,4-triazole reference material (purity: 100%)	9.94	10	1,2,4-triazole	994
TA stock solution		Triazole-alanine reference material (purity: 98.3%)	10.17	10	Triazole-alanine	999.7
TLA stock solution		Triazole-lactic acid hydrochloride reference material (purity: 78.5%)	15.10	10	Triazole-lactic acid	961.8*
TAA stock solution		Triazole-acetic acid reference material (purity: 95.7%)	10.05	10	Triazole-acetic acid	961.8
1,2,4-TRZ stock solution	27/09/2021	1,2,4-triazole reference material (purity: 100%)	9.98	10	1,2,4-triazole	998
TA stock solution		Triazole-alanine reference material (purity: 98.3%)	10.16	10	Triazole-alanine	998.7
TLA stock solution		Triazole-lactic acid hydrochloride reference material (purity: 78.5%)	15.07	10	Triazole-lactic acid	960.2*
TAA stock solution		Triazole-acetic acid reference material (purity: 95.7%)	10.21	10	Triazole-acetic acid	977.1
1,2,4-TRZ stock solution	11/11/2021	1,2,4-triazole reference material (purity: 100%)	11.16	10	1,2,4-triazole	1116
TA stock solution		Triazole-alanine reference material (purity: 98.3%)	10.68	10	Triazole-alanine	1050
TLA stock solution		Triazole-lactic acid hydrochloride reference material (purity: 78.5%)	14.18	10	Triazole-lactic acid	903.8*

TAA stock solution	Triazole-acetic acid reference material (purity: 95.7%)	10.24	10	Triazole-acetic acid	980.0
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* Concentration expressed as free acid (MW triazole lactic acid: 157.13 g/mol, MW triazole lactic acid HCl: 193.59 g/mol)

Internal standard stock solutions	Preparation date (dd/mm/yyyy)	Starting material	Weight (mg)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
TRZ ISTD stock solution	19/08/2021 20/09/2021	1,2,4-triazole[13C2,15N3] reference material (purity: 98.4%)	1.01	1	1,2,4-triazole[13C2,15N3]	994
TA ISTD stock solution		Triazole-alanine D2 reference material (purity: 95%)	1.01	1	Triazole-alanine D2	960
TLA ISTD stock solution		Triazole[13C2,15N3] lactic acid reference material (purity: 98.42%)	1.04	1	Triazole[13C2,15N3] lactic acid	1024
TAA ISTD stock solution		Triazole[13C2,15N3] acetic acid reference material (purity: 98.03%)	1.04	1	Triazole[13C2,15N3] acetic acid	1020

Diluted Solution	Starting material	Volume (μL)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
TDM Mix Solution A	1,2,4-TRZ stock solution	100*	10	1,2,4-triazole	10.0
	TA stock solution	100*		Triazole-alanine	10.0
	TLA stock solution	100*		Triazole-lactic acid	10.0
	TAA stock solution	100*		Triazole-acetic acid	10.0
TDM Mix Solution B	TDM Mix Solution A	1000	10	1,2,4-triazole	1.00
				Triazole-alanine	1.00
				Triazole-lactic acid	1.00
				Triazole-acetic acid	1.00
TDM Mix Solution C	TDM Mix Solution A	100	10	1,2,4-triazole	0.100
				Triazole-alanine	0.100
				Triazole-lactic acid	0.100
				Triazole-acetic acid	0.100
TDM MIX	TRZ ISTD stock solution	20	10	1,2,4-triazole[13C2,15N3]	1.99
	TA ISTD stock solution	21		Triazole-alanine D2	2.02
	TLA ISTD stock solution	20		Triazole[13C2,15N3] lactic acid	2.05
	TAA ISTD stock solution	20		Triazole[13C2,15N3] acetic acid	2.04

* Depending on the actual stock solution concentration, an appropriate volume was diluted in 10 mL of water to obtain a concentration of 10 µg/L of each analyte.

Matrix-matched analytical standard solutions for whole plant (rapeseed) and rapeseed oil were prepared from TDM Mix Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Matrix-matched standard solutions (for whole plant (rapeseed), rapeseed oil)						
Solution	µL of TDM Mix Solution B	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	10	2	0.5	10	0.002
L2	2.5	5	1	2.5	10	0.010
L3	5 (or 10) ²	5	1	5 (or 10) ²	10	0.020 (or 0.040) ²
L4	25	5	1	25	10	0.100
L5	50	5	1	50	10	0.200

1: Calculated considering the nominal sample preparation (5.00 g to a final volume of 20.0 mL)

2: L3 with a different concentration was used for rapeseed oil. This level can be indifferently of 5 or 10 µg/L since its concentration has no impact on the extension of the calibration range

Analytical standard solutions for rapeseed seeds, grain (wheat), white bread (wheat) were prepared in solvent (water / 1% formic acid in methanol 50:50) on the basis of the following scheme:

Standard solutions in solvent (for rapeseed seeds, grain (wheat), white bread (wheat))						
Solution	µL of TDM Mix Solution B	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	10	2	0.5	10	0.002
L2	2.5	5	1	2.5	10	0.010
L3	10	5	1	10	10	0.040
L4	25	5	1	25	10	0.100
L5	50	5	1	50	10	0.200

1: Calculated considering the nominal sample preparation (5.0 g to a final volume of 20.0 mL)

Standard solutions in solvent (for beer (barley))						
Solution	µL of TDM Mix Solution B	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	5	1	1	10	0.002
L2	5	5	1	5	10	0.010
L3	10	5	1	10	10	0.020
L4	50	5	1	50	10	0.100
L5	100	5	1	100	10	0.200

1: Calculated considering the nominal sample preparation (10.0 g to a final volume of 20.0 mL)

Standard solutions in solvent (for straw (wheat))						
Solution	µL	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	3.5 of TDM Mix Solution C	5	1	0.35	10	0.0028

L2	1 of TDM Mix Solution B	5	1	1.00	10	0.0080
L3	5 of TDM Mix Solution B	5	1	5.00	10	0.040
L4	1 of TDM Mix Solution A	5	1	10.0	10	0.080
L5	3.5 of TDM Mix Solution A	5	1	35.0	10	0.280

1: Calculated considering the nominal sample preparation (2.5 g to a final volume of 20.0 mL)

Furthermore, the linearity in solvent in a more concentrated calibration range was verified (from 10 to 1000 µg/L). The preparation of the working standard solution used for this purpose is reported in the following table:

Solution	Standard solutions in solvent					
	µL of TDM Mix Solution A	µL of TDM ISTD MIX	Final volume (mL)	TDM con- centration (each ana- lyte) (µg/L)	ISTD con- centration (each com- pound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	5	1	10	10	0.040
L2	5	5	1	50	10	0.200
L3	10	5	1	100	10	0.400
L4	50	5	1	500	10	2.00
L5	100	5	1	1000	10	4.00

1: Calculated considering a generic nominal sample preparation of 5.0 g to a final volume of 20.0 mL

The analyses were carried out using a HPLC-MS/MS system according to the following conditions:

Instrument: HPLC Shimadzu LC-40 XR + Sciex API 6500 + equipped with SelexION+ (Differential Ion Mobility Device)

Column: Thermo Hypercarb 5 µm, 2.1 x 100 mm

Column temperature: 40°C

Flow: 0.6 mL/min

Injection volume: 2 µL

Mobile phase A: LC-MS grade water with 1 % acetic acid

Mobile phase B: LC-MS grade methanol with 1 % acetic acid

Elution: gradient of the following composition:

Time (min)	% A	% B
0	95	5
5	10	90
6	10	90
6.1	95	5

Stop time: 10 min

Source type: ESI

Curtain gas flow: 30 mL/min

Gas 1: 55 mL/min

Gas 2: 65 mL/min

Gas temperature: 500°C

Capillary: positive mode 3500 V

Acquiring mode: ESI positive, MRM (multi-reaction monitoring).

Calibration

The quantification of each analyte was made through the building of a calibration straight line with the internal standard method. 5 analytical standard solutions for each analysed matrix were analysed in single

injections in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy) and Repeatability (Precision)

Recovery and repeatability (as precision, % RSD) data will be reported for the following fortification levels:

- LOQ level (5 replicates): 0.01 mg/kg
- 10xLOQ level (5 replicates): 0.1 mg/kg - or alternatively at a spiking level higher than maximum residue level found on field specimens.

Specificity (Selectivity)

This parameter will be evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by matrix interferences.

Using a differential ion mobility device and a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: samples, fortified samples, reference solutions at the LOQ level, in order to assess the presence or absence of interfering signals. Blank values (non-fortified samples) will be determined from the matrices used in fortification experiments and should not be higher than 30% of the LOQ.

Matrix effect

Assessment of matrix effects will be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix, for all sample matrix used in the study. In order to nullify matrix effect, calibration curves for all matrices analysed will be prepared using matrix matched analytical standards.

Stability of final extracts and standard

The samples extracts will be preferably injected the same day of the extraction, if not analysed immediately they will be stored at $5 \pm 3^{\circ}\text{C}$ in dark conditions.

Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample. It is the lowest concentration at which an analyte produces an instrumental signal at least 3 times higher than background noise of the chromatogram. It should be not higher than 30% of LOQ value and it will be considered as the lowest point of the instrumental calibration.

Limit of quantification (LOQ)

Limit of quantitation (LOQ) is defined as the lowest validated level with sufficient recovery and precision. Target LOQ for Triazole Derivative Metabolites (TDMs) in the sample analysed will be set to 0.01 mg/kg.

Confirmation:

A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring an additional SRM transition. The following data will be provided for the additional ion: calibration data as recorded for primary detection, recovery and precision data as recorded for primary detection (at least for the 5 replicates at LOQ level) the recovery and precision mean values calculated on confirmatory ion must fulfill the same acceptable range reported above for primary detection.

Results and discussions

Method validation data can be summarised in the tables below.

Table A 6: Recovery results from method validation of 1,2,4-triazole (1,2,4-TRZ) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	87.0	6.2	7.2	
		0.1 (10xLOQ)	98.7	4.0	4.1	
Rapeseed seeds	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	99.0	2.3	2.3	
		0.1 (10xLOQ)	93.9	4.0	4.3	
Wheat grain	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	87.5	7.5	8.6	
		0.1 (10xLOQ)	97.7	5.3	5.4	
Straw (wheat)	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	93.9	6.2	6.6	
		0.1 (10xLOQ)	95.6	2.1	2.2	
Rapeseed oil	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	94.2	7.9	8.4	
		0.1 (10xLOQ)	98.4	2.4	2.5	
White bread (wheat)	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	97.7	5.3	5.5	
		0.1 (10xLOQ)	98.7	3.4	3.4	
Beer (barley)	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	102.6	6.3	6.2	
		0.1 (10xLOQ)	100.6	3.1	3.1	

Table A 7: Recovery results from method validation of Triazole-alanine (TA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	Triazole-alanine (TA)	0.01(LOQ)	98.7	2.9	2.9	
		0.1 (10xLOQ)	99.5	2.3	2.3	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Rapeseed seeds	Triazole-alanine (TA)	0.01(LOQ)	102.0	4.7	4.6	
		0.1 (10xLOQ)	112.0	1.8	1.6	
Wheat grain	Triazole-alanine (TA)	0.01(LOQ)	91.2	5.7	6.3	
		0.1 (10xLOQ)	106.5	1.6	1.5	
Straw (wheat)	Triazole-alanine (TA)	0.01(LOQ)	100.0	5.2	5.2	
		0.1 (10xLOQ)	99.0	3.8	3.8	
Rapeseed oil	Triazole-alanine (TA)	0.01(LOQ)	100.6	1.5	1.5	
		0.1 (10xLOQ)	100.0	1.5	1.5	
White bread (wheat)	Triazole-alanine (TA)	0.01(LOQ)	100.3	2.5	2.5	
		0.1 (10xLOQ)	100.7	3.4	3.3	
Beer (barley)	Triazole-alanine (TA)	0.01(LOQ)	104.1	13.0	12.5	
		0.1 (10xLOQ)	109.4	5.2	4.8	

Table A 8: Recovery results from method validation of Triazole-lactic acid (TLA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	Triazole-lactic acid (TLA)	0.01(LOQ)	101.3	1.8	1.8	
		0.1 (10xLOQ)	99.8	1.5	1.5	
Rapeseed seeds	Triazole-lactic acid (TLA)	0.01(LOQ)	113.0	1.7	1.5	
		0.1 (10xLOQ)	106.6	0.9	0.8	
Wheat grain	Triazole-lactic acid (TLA)	0.01(LOQ)	104.2	3.6	3.4	
		0.1 (10xLOQ)	104.6	2.0	1.9	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Straw (wheat)	Triazole-lactic acid (TLA)	0.01(LOQ)	99.5	4.8	4.8	
		0.1 (10xLOQ)	99.7	1.2	1.2	
Rapeseed oil	Triazole-lactic acid (TLA)	0.01(LOQ)	100.2	2.4	2.4	
		0.1 (10xLOQ)	97.7	1.2	1.2	
White bread (wheat)	Triazole-lactic acid (TLA)	0.01(LOQ)	99.9	3.8	3.8	
		0.1 (10xLOQ)	100.3	1.0	1.0	
Beer (barley)	Triazole-lactic acid (TLA)	0.01(LOQ)	97.6	3.0	3.1	
		0.1 (10xLOQ)	97.5	3.7	3.8	

Table A 9: Recovery results from method validation of Triazole-acetic acid (TAA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Whole plant (rapeseed)	Triazole-acetic acid (TAA)	0.01(LOQ)	100.8	1.7	1.7	
		0.1 (10xLOQ)	100.0	1.3	1.3	
Rapeseed seeds	Triazole-acetic acid (TAA)	0.01(LOQ)	102.9	3.3	3.2	
		0.1 (10xLOQ)	99.1	1.3	1.3	
Wheat grain	Triazole-acetic acid (TAA)	0.01(LOQ)	100.0	4.2	4.2	
		0.1 (10xLOQ)	101.9	2.2	2.1	
Straw (wheat)	Triazole-acetic acid (TAA)	0.01(LOQ)	98.9	6.6	6.7	
		0.1 (10xLOQ)	102.2	1.0	1.0	
Rapeseed oil	Triazole-acetic acid (TAA)	0.01(LOQ)	103.0	1.7	1.6	
		0.1 (10xLOQ)	99.4	0.8	0.8	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
White bread (wheat)	Triazole-acetic acid (TAA)	0.01(LOQ)	101.1	2.5	2.5	
		0.1 (10xLOQ)	98.5	0.9	0.9	
Beer (barley)	Triazole-acetic acid (TAA)	0.01(LOQ)	100.1	17.5	17.5	
		0.1 (10xLOQ)	103.1	1.6	1.6	

Table A 10: Characteristics for the analytical method used for validation of TDMs residues in wheat, barley, oilseed rape and processed commodities

	1,2,4-triazole (1,2,4-TRZ)	Triazole alanine (TA)	Triazole lactic acid (TLA)	Triazole acetic acid (TAA)
Specificity	MS spectrum provided: Yes m/z 70.1 – 43.1 m/z 70.1 – 70.0	MS spectrum provided: Yes m/z 157.0 – 70.0 m/z 157.0 – 88.0	MS spectrum provided: Yes m/z 158.0 – 70.0 m/z 158.0 – 43.0	MS spectrum provided: Yes m/z 128.0 – 70 m/z 128.0 – 73.0
Calibration (type, number of data points)	Whole Plant (Rapeseed): n = 5 y= 1.03477x + 0.07821 R ² = 0.999	Whole Plant (Rapeseed): n = 5 y= 0.51285x + 0.02730 R ² = 0.999	Whole Plant (Rapeseed): n = 5 y= 1.20823 x + 0.02456 R ² = 0.999	Whole Plant (Rapeseed): n = 5 y= 0.98409x + 0.01569 R ² = 0.999
	Rapeseed oil: n = 5 y = 0.94482x + 0.03984 R ² = 0.999	Rapeseed oil: y = 2.37172x + 0.01580 R ² = 0.999	Rapeseed oil: n = 5 y = 1.22940 x + - 0.00528 R ² = 0.999	Rapeseed oil: y = 0.098796x + 1.07526e-4 R ² = 0.999
	Rapeseed seeds/Grain (wheat)/White bread (wheat): n = 5 y = 0.89935x + 0.04423 R ² = 0.999	Rapeseed seeds/Grain(wheat)/ White bread (wheat): n = 5 y = 2.76713 x + 0.02212 R ² = 0.999	Rapeseed seeds/Grain(wheat)/ White bread (wheat): n = 5 y = 1.02675x +- 9.58870e-4 R ² = 0.999	Rapeseed seeds/Grain(wheat)/ White bread (wheat): n = 5 y = 1.00664 x + 0.00803 R ² = 0.999
	Straw (wheat) n = 5 y = 0.97019 x + 0.00769 R ² = 0.999	Straw (wheat) n = 5 y = 5.15827 x + 0.09160 R ² = 0.999	Straw (wheat) n = 5 y = 1.19902 x + 0.01208 R ² = 0.999	Straw (wheat) n = 5 y = 1.03861 x + 4.1612e-4 R ² = 0.999

	Beer (barley) n = 5 $y = 0.94954 x + 0.00154$ $R^2 = 0.999$	Beer (barley) n = 5 $y = 3.83597 x + 0.16176$ $R^2 = 0.999$	Beer (barley) n = 5 $y = 1.31066 x + 0.07797$ $R^2 = 0.999$	Beer (barley) n = 5 $y = 0.95844 x + 0.01940$ $R^2 = 0.999$
Calibration range	Range: 0.50 – 50.0 µg/L (0.00200 – 0.200mg/kg) Beer range: 1.00 – 100 µg/L (0.0020 – 0.200 mg/kg) Straw (wheat) range: 0.35 – 35.0µg/L (0.0028 – 0.280mg/kg)	Range: 0.50 – 50.0 µg/L (0.00200 – 0.200mg/kg) Beer range: 1.00 – 100 µg/L (0.0020 – 0.200 mg/kg) Straw (wheat) range: 0.35 – 35.0µg/L (0.0028 – 0.280mg/kg)	Range: 0.50 – 50.0 µg/L (0.00200 – 0.200mg/kg) Beer range: 1.00 – 100 µg/L (0.0020 – 0.200 mg/kg) Straw (wheat) range: 0.35 – 35.0µg/L (0.0028 – 0.280mg/kg)	Range: 0.50 – 50.0 µg/L (0.00200 – 0.200mg/kg) Beer range: 1.00 – 100 µg/L (0.0020 – 0.200 mg/kg) Straw (wheat) range: 0.35 – 35.0µg/L (0.0028 – 0.280mg/kg)
Assessment of matrix effects is presented	Yes Except for triazole-alanine in whole plant, the matrix effect is considered not significant according to the SANTE/2020/12830 rev.1 guideline, since the analytes responses in matrix matched calibration solutions are $< \pm 20\%$ of the analytes responses in the calibration solutions prepared in solvent (water/methanol 50:50 + 1% formic acid). Since the matrix effect is negligible, the calibration can also be done using standards prepared in solvent.			
Limit of determination/quantification	LOD: 0.50 µg/L LOD: 1 µg/L for beer LOD for wheat: 0.35 µg/L LOQ: 0.01 mg/kg	LOD: 0.50 µg/L LOD: 1 µg/L for beer LOD for wheat: 0.35 µg/L LOQ: 0.01 mg/kg	LOD: 0.50 µg/L LOD: 1 µg/L for beer LOD for wheat: 0.35 µg/L LOQ: 0.01 mg/kg	LOD: 0.50 µg/L LOD: 1 µg/L for beer LOD for wheat: 0.35 µg/L LOQ: 0.01 mg/kg

Conclusion

A mean recovery of 60-120% with a Relative Standard Deviation $\leq 30\%$ was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.1.3.2 ILV Method for whole plant (rapeseed) in TDMs

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2
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	This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/06

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed)
Rigamonti, E. 2022
Study No. GLP-STUDY-1085/2021

Guideline(s): Yes.
- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Postapproval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-108 (KCP 5.2.1-05) for the determination of triazole-derivative metabolites (TDMs) in whole plant (Rapeseed) in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 5 mL of water and 10.0 mL of formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The separation of the organic phase was achieved by centrifugation. 1.0 mL of the supernatant was taken and filtered using PVDF syringe filter at 0.45 µm.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Repeatability and recovery tests were performed using fortified Whole Plant (Rapeseed) samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration.

The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ.

No interferences above the LOD were found in the control Whole Plant (Rapeseed) samples, the analysis of fortified samples at low and high level gave the following results

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
1,2,4-triazole (1,2,4-TRZ)	43.1	Low	0.011	5	0.009	87.5	5.23	0.0
		High	0.105	5	0.101	97.1	3.03	0.0
1,2,4-triazole (1,2,4-TRZ)	70.0	Low	0.011	5	0.010	93.6	2.63	0.0
		High	0.105	5	0.103	99.0	3.69	0.0
Triazole alanine (TA)	70.0	Low	0.008	5	0.007	89.8	10.03	0.0
		High	0.075	5	0.075	100.4	4.04	0.0
Triazole alanine (TA)	88.0	Low	0.008	5	0.007	98.5	8.83	0.0
		High	0.075	5	0.076	102.5	3.61	0.0

Triazole lactic acid (TLA)	70.0	Low	0.010	5	0.009	97.1	2.29	0.0
		High	0.096	5	0.096	100.4	0.97	0.0
Triazole lactic acid (TLA)	43.0	Low	0.010	5	0.010	100.8	4.20	0.0
		High	0.096	5	0.097	101.6	1.49	0.0
Triazole acetic acid (TAA)	70.0	Low	0.010	5	0.010	99.6	2.11	0.0
		High	0.105	5	0.105	101.8	0.85	0.0
Triazole acetic acid (TAA)	73.0	Low	0.010	5	0.010	92.7	7.72	0.0
		High	0.105	5	0.104	100.3	2.81	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
1,2,4-triazole (1,2,4-TRZ)	43.1	87.5	5.23	97.1	3.03	92.3	6.76
	70.0	93.6	2.63	99.0	3.69	96.3	4.25
Triazole alanine (TA)	70.0	89.8	10.03	100.4	4.04	95.1	9.07
	88.0	98.5	8.83	102.5	3.61	100.5	6.62
Triazole lactic acid (TLA)	70.0	97.1	2.29	100.4	0.97	98.7	2.43
	43.0	100.8	4.20	101.6	1.49	101.2	2.99
Triazole acetic acid (TAA)	70.0	99.6	2.11	101.8	0.85	100.7	1.90
	73.0	92.7	7.72	100.3	2.81	96.5	6.74

Since all recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify TDMs in Whole Plant (Rapeseed) samples with an established L.O.Q of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC-DMS-MS/MS instrument with quantification by internal standard, was shown to be specific for TDMs (triazole-derivative metabolites) residues in Whole Plant (Rapeseed).

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
1,2,4-triazole (1,2,4-TRZ)	70.1	Quantifier	43.1	-10
Triazole alanine (TA)	157.0	Quantifier	70.0	-7
Triazole lactic acid (TLA)	158.0	Quantifier	70.0	3
Triazole acetic acid (TAA)	128.0	Quantifier	70.0	1

Not significant matrix effects for all TDMs (triazole-derivative metabolites) residues in Whole Plant (Rapeseed) matrix were found (< ± 20%). Therefore, the calibration standards could be prepared in solvent or in matrix. Matrix-matched calibration standards were used throughout the entire study.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ. The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for each analyte, corresponding to about 0.002 mg/kg in Whole Plant (Rapeseed) (30% of the LOQ). The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (60 – 120% with % RSD < 30%), was a final injected solution of about 2.50 µg/L for each analyte, corresponding to about 0.010 mg/kg in Whole Plant (Rapeseed).

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
1,2,4-triazole (1,2,4-TRZ)	0.50	0.002	2.50	0.010
Triazole alanine (TA)	0.50	0.002	2.50	0.010
Triazole lactic acid (TLA)	0.50	0.002	2.50	0.010
Triazole acetic acid (TAA)	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Confirmation

Since the analysis by HPLC using internal standards and MS triple quadrupole detector (HPLC/MS/MS) equipped with a Differential Mobility Separation (DMS) device in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole (1,2,4-TRZ)	quantifier	70.1	43.1
	qualifier		70.0
Triazole alanine (TA)	quantifier	157.0	70.0
	qualifier		88.0
Triazole lactic acid (TLA)	quantifier	158.0	70.0
	qualifier		43.0
Triazole acetic acid (TAA)	quantifier	128.0	70.0
	qualifier		73.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in rapeseed with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.3.3 ILV Method for rapeseed in TDMs

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/07

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Rapeseed seeds
Rigamonti, E. 2022
Study No. GLP-STUDY-1090/2021

Guideline(s): Yes.
- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Postapproval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-108 (KCP 5.2.1-05) for the determination of triazole-derivative metabolites (TDMs) in Rapeseed seeds in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 100 µL of TDM ISTD MIX and 10.0 mL of water were added. In a second time, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The separation of the organic phase was achieved by centrifugation. 1.0 mL of the supernatant was taken and filtered using PVDF syringe filter at 0.45 µm.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using fortified Rapeseed seeds samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration.

Since no matrix sample (Rapeseed seeds) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
1,2,4-triazole (1,2,4-TRZ)	43.1	Low	0.011	5	0.010	102.7	3.88	0.0
		High	0.105	5	0.104	102.3	3.16	0.0
1,2,4-triazole (1,2,4-TRZ)	70.0	Low	0.011	5	0.011	104.2	4.62	0.0
		High	0.105	5	0.105	102.7	2.24	0.0
Triazole alanine (TA)	70.0	Low	0.008	5	0.007	93.4	9.51	0.0
		High	0.075	5	0.068	96.9	4.54	0.0
Triazole alanine (TA)	88.0	Low	0.008	5	0.007	98.5	7.13	0.0
		High	0.075	5	0.072	101.8	4.50	0.0
Triazole lactic acid (TLA)	70.0	Low	0.010	5	0.011	107.5	1.39	0.0
		High	0.096	5	0.102	103.6	1.71	0.0
Triazole lactic acid (TLA)	43.0	Low	0.010	5	0.010	104.5	4.54	0.0
		High	0.096	5	0.104	105.8	0.96	0.0
Triazole acetic acid (TAA)	70.0	Low	0.010	5	0.010	100.7	1.88	0.0
		High	0.105	5	0.105	104.1	1.07	0.0
Triazole acetic acid (TAA)	73.0	Low	0.010	5	0.009	94.0	7.91	0.0
		High	0.105	5	0.107	105.9	3.30	0.0

Matrix dried	Product	Spike Low or LOQ	Spike High or 10 x	Overall
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tomatoes	ion	(n = 5)		LOQ (n = 5)		(n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
1,2,4-triazole (1,2,4-TRZ)	43.1	102.7	3.88	102.3	3.16	102.5	3.34
	70.0	104.2	4.62	102.7	2.24	103.5	3.52
Triazole alanine (TA)	70.0	93.4	9.51	96.9	4.54	95.2	7.21
	88.0	98.5	7.13	101.8	4.50	100.1	5.85
Triazole lactic acid (TLA)	70.0	107.5	1.39	103.6	1.71	105.5	2.44
	43.0	104.5	4.54	105.8	0.96	105.2	3.14
Triazole acetic acid (TAA)	70.0	100.7	1.88	104.1	1.07	102.4	2.29
	73.0	94.0	7.91	105.9	3.30	100.0	8.35

Since all recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify TDMs (triazole-derivative metabolites) in Rapeseed seeds samples with an established LOQ of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC-DMS-MS/MS instrument with quantification by internal standard, was shown to be specific for TDMs (triazole-derivative metabolites) residues in Rapeseed seeds.

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
1,2,4-triazole (1,2,4-TRZ)	70.1	Quantifier	43.1	1
Triazole alanine (TA)	157.0	Quantifier	70.0	-19
Triazole lactic acid (TLA)	158.0	Quantifier	70.0	0
Triazole acetic acid (TAA)	128.0	Quantifier	70.0	1

Since no matrix sample free from the Triazole-alanine analyte was available and the matrix effect was not significant ($< \pm 20\%$), it was necessary to prepare the calibration standard solution in solvent.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ.

The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for each analyte, corresponding to about 0.002 mg/kg in Rapeseed seeds (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (60 – 120% with % RSD < 30%), was a final injected solution of about 2.50 µg/L for each analyte, corresponding to about 0.010 mg/kg in Rapeseed seeds.

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
1,2,4-triazole (1,2,4-TRZ)	0.50	0.002	2.50	0.010

Triazole alanine (TA)	0.50	0.002	2.50	0.010
Triazole lactic acid (TLA)	0.50	0.002	2.50	0.010
Triazole acetic acid (TAA)	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Confirmation

Since the analysis by HPLC using internal standards and MS triple quadrupole detector (HPLC/MS/MS) equipped with a Differential Mobility Separation (DMS) device in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole (1,2,4-TRZ)	quantifier	70.1	43.1
	qualifier		70.0
Triazole alanine (TA)	quantifier	157.0	70.0
	qualifier		88.0
Triazole lactic acid (TLA)	quantifier	158.0	70.0
	qualifier		43.0
Triazole acetic acid (TAA)	quantifier	128.0	70.0
	qualifier		73.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in rapeseed seeds with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.4 Analytical method 4

A 2.1.1.1.4.1 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/08

Report Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in apple, carrot, tomato and processed commodities
 Longhi, D. 2021
 Study No. GLP-STUDY-21-109

Guideline(s): Yes.

- European Commission, Guidance Document on Pesticide Analytical

Methods for Risk Assessment and Postapproval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

- European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.
- OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17.
- “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPE-PO-Method)- Method 8 (M8)”

Deviations: No
GLP: Yes
Acceptability: Yes

Materials and methods

The validation of the analytical method was carried out under GLP compliance to SANTE/2020/12830 Rev.1 guideline. The analytical determination was carried out using a HPLC-MS/MS method validated in the GLP study coded GLP-STUDY-21-109.

Sample extraction

Aliquots of 5 or 10 g of specimen were taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of 100 µL of the internal standard solution TDM ISTD MIX (2 mg/L of each internal standard) and by the following amounts of deionized water (added on the basis of QuPPE-PO-Method and considering the theoretical water content of each matrix):

Matrix	Sample weight (g)	Water added (mL)
Apple	10	1.5
Tomato	10	0.5
Carrot	10	1
Dried apple	5	9
Dried tomato	5	9
Apple juice	10	1

Then, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The volume of the final extract is considered to be 20 mL: little variation due to the actual water content of each sample are corrected by the presence of the internal standard, that is added to produce a concentration in the final extract nominally of 10 µg/L of each compound. The separation of the liquid phase from the solid one was achieved by centrifugation at 5000 rpm for 5 minutes. An aliquot of about 1 mL the supernatant was taken, filtered with a 0.45 µm PVDF filter and transferred in a 2 mL HPLC glass vial for the final analysis with a HPLC-DMS-MS/MS system.

Reference solutions preparation:

The following reference solutions were prepared:

Stock solution	Starting material	Weight/Volume	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
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1,2,4-TRZ stock solution	1,2,4-triazole reference material (purity: 100%)	9.94 mg	10	1,2,4-triazole	994
TA stock solution	Triazole-alanine reference material (purity: 98.3%)	10.17 mg	10	Triazole-alanine	999.7
TLA stock solution	Triazole-lactic acid hydrochloride reference material (purity: 78.5%)	15.10 mg	10	Triazole-lactic acid	1185*
TAA stock solution	Triazole-acetic acid reference material (purity: 95.7%)	10.05 mg	10	Triazole-acetic acid	961.8

* Concentration expressed as free acid (MW triazole lactic acid: 157.13 g/mol, MW triazole lactic acid HCl: 193.59 g/mol)

Internal standard stock Solution	Starting material	Weight/Volume	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
TRZ ISTD stock solution	1,2,4-triazole[¹³ C ₂ , ¹⁵ N ₃] reference material (purity: 98.4%)	1.01 mg	10	1,2,4-triazole [¹³ C ₂ , ¹⁵ N ₃]	994
TA ISTD stock solution	Triazole-alanine D ₂ reference material (purity: 95%)	1.01 mg	10	Triazole-alanine D ₂	960
TLA ISTD stock solution	Triazole[¹³ C ₂ , ¹⁵ N ₃] lactic acid reference material (purity: 98.42%)	1.04 mg	10	Triazole[¹³ C ₂ , ¹⁵ N ₃]-lactic acid	1024
TAA ISTD stock solution	Triazole[¹³ C ₂ , ¹⁵ N ₃] acetic acid reference material (purity: 98.03%)	1.04 mg	10	Triazole[¹³ C ₂ , ¹⁵ N ₃]-acetic acid	1020

Diluted Solution	Starting material	Volume (µL)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)
TDM Mix Solution A	1,2,4-TRZ stock solution	101	10	1,2,4-triazole	10.0
	TA stock solution	100		Triazole-alanine	10.0
	TLA stock solution	104		Triazole-lactic acid	10.0
	TAA stock solution	104		Triazole-acetic acid	10.0
TDM Mix Solution B	TDM Mix Solution A	1000 mg	10	1,2,4-triazole	1.00
				Triazole-alanine	1.00
				Triazole-lactic acid	1.00
				Triazole-acetic acid	1.00
TDM ISTD MIX	TRZ ISTD stock solution	20	10	1,2,4-triazole[13C2, 15N3]	1.99
	TA ISTD stock solution	21		Triazole-alanine D2	2.02
	TLA ISTD stock solution	20		Triazole[13C2, 15N3] lactic acid	2.05
	TAA ISTD stock solution	20		Triazole[13C2, 15N3] acetic acid	2.04

Matrix-matched analytical standard solutions for apple, tomato, carrot, apple juice were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of TDM Solution B	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	5	1	1	10	0.002
L2	5	5	1	5	10	0.010
L3	10	5	1	10 (or 20) ²	10	0.020(or 0.040) ²
L4	50	5	1	50	10	0.100
L5	100	5	1	100	10	0.200

1: Calculated considering the nominal sample preparation (10.0 g to a final volume of 20.0 mL)

2: L3 with a different concentration was used for tomato and apple juice. This level can be indifferently of 10 or 20 µg/L since its concentration has no impact on the extension of the calibration range

Matrix-matched analytical standard solutions for dried apple, dried tomato were prepared from Solution B using the final extract of an unfortified aliquot on the basis of the following scheme:

Solution	µL of TDM Solution B	µL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (µg/L)	ISTD concentration (each compound) (µg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	10	2	0.5	10	0.002
L2	2.5	5	1	2.5	10	0.010
L3	10	5	1	10	10	0.020
L4	25	5	1	25	10	0.100
L5	50	5	1	50	10	0.200

1: Calculated considering the nominal sample preparation (5.0 g to a final volume of 20.0 mL)

The analyses were carried out using a HPLC-MS/MS system according to the following conditions:

Instrument: HPLC Shimadzu LC-40 XR + Sciex API 6500 + equipped with SelexION+ (Differential Ion Mobility Device)

Column: Thermo Hypercarb 5 µm, 2.1 x 100 mm

Column temperature: 40°C

Flow: 0.6 mL/min

Injection volume: 2 µL

Mobile phase A: LC-MS grade water with 1 % acetic acid

Mobile phase B: LC-MS grade methanol with 1 % acetic acid

Elution: gradient of the following composition:

Time (min)	% A	% B
0	95	5
5	10	90
6	10	90
6.1	95	5

Stop time: 10 min

Source type: ESI

Curtain gas flow: 30 mL/min

Gas 1: 55 mL/min

Gas 2: 65 mL/min

Gas temperature: 500°C

Capillary: positive mode 3500 V

Acquiring mode: ESI positive, MRM (multi-reaction monitoring).

Calibration

The quantification of each analyte was made through the building of a calibration straight line with the internal standard method. 5 analytical standard solutions for each analysed matrix were analysed in single injections in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy) and Repeatability (Precision)

Recovery and repeatability (as precision, % RSD) data will be reported for the following fortification levels:

- LOQ level (5 replicates): 0.01 mg/kg
- 10xLOQ level (5 replicates): 0.1 mg/kg - or alternatively at a spiking level higher than maximum residue level found on field specimens.

Specificity (Selectivity)

This parameter will be evaluated in order to demonstrate that the applied method detects the right analyte and that the analytical signal is quantitatively correct and not affected by other analytes or by matrix interferences.

Using a differential ion mobility device and a MS/MS mass spectrometer detector the selectivity was evaluated comparing the following chromatograms: samples, fortified samples, reference solutions at the LOQ level, in order to assess the presence or absence of interfering signals. Blank values (non-fortified samples) will be determined from the matrices used in fortification experiments and should not be higher than 30% of the LOQ.

Matrix effect

Assessment of matrix effects will be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix, for all sample matrix used in the study. In order to nullify matrix effect, calibration curves for all matrices analysed will be prepared using matrix matched analytical standards.

Stability of final extracts and standard

The samples extracts will be preferably injected the same day of the extraction, if not analysed immediately they will be stored at $5 \pm 3^{\circ}\text{C}$ in dark conditions.

Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample. It is the lowest concentration at which an analyte produces an instrumental signal at least 3 times higher than background noise of the chromatogram. It should be not higher than 30% of LOQ value and it will be considered as the lowest point of the instrumental calibration.

Limit of quantification (LOQ)

Limit of quantitation (LOQ) is defined as the lowest validated level with sufficient recovery and precision. Target LOQ for Triazole Derivative Metabolites (TDMs) in the sample analysed will be set to 0.01 mg/kg.

Confirmation:

A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring an additional SRM transition. The following data will be provided for the additional ion: calibration data as recorded for primary detection, recovery and precision data as recorded for primary detection (at least for the 5 replicates at LOQ level) the recovery and precision mean values calculated on confirmatory ion must fulfill the same acceptable range reported above for primary detection.

Results and discussions

Method validation data can be summarised in the tables below.

Table A 11: Recovery results from method validation of 1,2,4-triazole (1,2,4-TRZ) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) ($n = x$)	Mean recovery (%)	SD	RSD (%)	Comments
Apple	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	101.1	1.3	1.2	
		0.1 (10xLOQ)	102.5	0.38	0.37	
Tomato	1,2,4-triazole	0.01(LOQ)	96.6	1.2	1.3	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
	(1,2,4-TRZ)	0.1 (10xLOQ)	99.9	2.1	2.1	
Carrot	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	99.2	2.9	3.0	
		0.1 (10xLOQ)	97.8	2.1	2.1	
Dried apple	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	100.7	3.3	3.3	
		0.1 (10xLOQ)	98.0	2.0	2.0	
Apple juice	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	100.2	2.0	2.0	
		0.1 (10xLOQ)	100.4	1.7	1.7	
Dried tomato	1,2,4-triazole (1,2,4-TRZ)	0.01(LOQ)	95.8	3.4	3.6	
		0.1 (10xLOQ)	100.2	1.6	1.6	

Table A 12: Recovery results from method validation of Triazole-alanine (TA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
Apple	Triazole-alanine (TA)	0.01(LOQ)	98.1	1.6	1.6	
		0.1 (10xLOQ)	97.9	2.8	2.9	
Tomato	Triazole-alanine (TA)	0.01(LOQ)	94.9	3.3	3.5	
		0.1 (10xLOQ)	100.5	2.1	2.1	
Carrot	Triazole-alanine (TA)	0.01(LOQ)	93.6	2.5	2.7	
		0.1 (10xLOQ)	100.3	0.44	0.44	
Dried apple	Triazole-alanine (TA)	0.01(LOQ)	99.8	4.3	4.3	
		0.1 (10xLOQ)	100.4	1.7	1.7	
Apple juice	Triazole-alanine (TA)	0.01(LOQ)	100.8	2.3	2.3	
		0.1 (10xLOQ)	106.3	1.1	1.0	
Dried	Triazole-	0.01(LOQ)	100.5	2.0	2.0	

Matrix	Analyte	Fortification level (mg/kg) ($n = x$)	Mean recovery (%)	SD	RSD (%)	Comments
tomato	alanine (TA)	0.1 (10xLOQ)	98.6	1.8	1.9	

Table A 13: Recovery results from method validation of Triazole-lactic acid (TLA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) ($n = x$)	Mean recovery (%)	SD	RSD (%)	Comments
Apple	Triazole-lactic acid (TLA)	0.01(LOQ)	99.7	2.4	2.4	
		0.1 (10xLOQ)	97.1	1.9	1.9	
Tomato	Triazole-lactic acid (TLA)	0.01(LOQ)	101.7	1.7	1.6	
		0.1 (10xLOQ)	103.3	1.6	1.6	
Carrot	Triazole-lactic acid (TLA)	0.01(LOQ)	100.0	2.0	2.0	
		0.1 (10xLOQ)	98.7	1.9	1.9	
Dried apple	Triazole-lactic acid (TLA)	0.01(LOQ)	101.4	1.4	1.4	
		0.1 (10xLOQ)	98.7	1.5	1.5	
Apple juice	Triazole-lactic acid (TLA)	0.01(LOQ)	98.6	1.5	1.5	
		0.1 (10xLOQ)	100.6	2.4	2.4	
Dried tomato	Triazole-lactic acid (TLA)	0.01(LOQ)	100.8	1.6	1.6	
		0.1 (10xLOQ)	101.3	1.7	1.7	

Table A 14: Recovery results from method validation of Triazole-acetic acid (TAA) using the analytical method

Matrix	Analyte	Fortification level (mg/kg) ($n = x$)	Mean recovery (%)	SD	RSD (%)	Comments
Apple	Triazole-acetic acid (TAA)	0.01(LOQ)	106.0	0.66	0.62	
		0.1 (10xLOQ)	101.6	1.3	1.3	
Tomato	Triazole-acetic	0.01(LOQ)	105.5	1.4	1.4	

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	SD	RSD (%)	Comments
	acid (TAA)	0.1 (10xLOQ)	101.0	1.2	1.2	
Carrot	Triazole-acetic acid (TAA)	0.01(LOQ)	101.6	1.4	1.3	
		0.1 (10xLOQ)	99.9	0.63	0.63	
Dried apple	Triazole-acetic acid (TAA)	0.01(LOQ)	99.9	1.2	1.2	
		0.1 (10xLOQ)	99.1	0.91	0.92	
Apple juice	Triazole-acetic acid (TAA)	0.01(LOQ)	108.1	2.3	2.1	
		0.1 (10xLOQ)	106.8	1.9	1.8	
Dried tomato	Triazole-acetic acid (TAA)	0.01(LOQ)	98.6	1.7	1.7	
		0.1 (10xLOQ)	101.0	0.64	0.63	

Table A 15: Characteristics for the analytical method used for validation of TDMs residues in apple, carrot, tomato and processed commodities

	1,2,4-triazole (1,2,4-TRZ)	Triazole alanine (TA)	Triazole lactic acid (TLA)	Triazole acetic acid (TAA)
Specificity	MS spectrum provided: Yes m/z 70.1 – 43.1 m/z 70.1 – 70.0	MS spectrum provided: Yes m/z 157.0 – 70.0 m/z 157.0 – 88.0	MS spectrum provided: Yes m/z 158.0 – 70.0 m/z 158.0 – 43.0	MS spectrum provided: Yes m/z 128.0 – 70 m/z 128.0 – 73.0
Calibration (type, number of data points)	Apple: n = 5 y = 0.90937x + 0.04113 R ² = 0.999	Apple: n = 5 y = 2.96833 x + 0.021516 R ² = 0.999	Apple: n = 5 y = 0.97727 x + 0.00789 R ² = 0.999	Apple: n = 5 y = 0.86083 x + 0.01170 R ² = 0.99
	Tomato: n = 5 y = 0.43772 x + 0.05088 R ² = 0.999	Tomato: n = 5 y = 1.65348 x + 0.34660 R ² = 0.999	Tomato: n = 5 y = 0.55522 x + 0.04599 R ² = 0.999	Tomato: n = 5 y = 0.48833 x + 0.00228 R ² = 0.999
	Carrot: n = 5 y = 0.10111 x + 0.03963 R ² = 0.999	Carrot: n = 5 y = 0.30741 x + 0.40990 R ² = 0.999	Carrot: n = 5 y = 0.09408 x + 0.06186 R ² = 0.999	Carrot: n = 5 y = 0.08351 x + 0.02735 R ² = 0.999
	Dried apple: n = 5 y = 0.97734 x + 0.00929 R ² = 0.999	Dried apple n = 5 y = 2.75226 x + 0.18048 R ² = 0.999	Dried apple n = 5 y = 1.02094 x + 0.01300 R ² = 0.999	Dried apple n = 5 y = 0.89384 x + 0.01211 R ² = 0.999

	Apple juice: n = 5 $y = 0.85741 x + 0.01792$ $R^2 = 0.999$	Apple juice: n = 5 $y = 3.23919 x + 0.41947$ $R^2 = 0.999$	Apple juice: n = 5 $y = 1.05690 x + 0.03702$ $R^2 = 0.999$	Apple juice: n = 5 $y = 0.85941 x + 0.01835$ $R^2 = 0.999$
	Dried tomato: n = 5 $y = 0.96328 x + 0.02864$ $R^2 = 0.999$	Dried tomato: n = 5 $y = 3.24108 x + 171413$ $R^2 = 0.999$	Dried tomato: n = 5 $y = 1.01765 x + 0.28693$ $R^2 = 0.999$	Dried tomato: n = 5 $y = 0.88868 x + 0.03273$ $R^2 = 0.999$
Calibration range	Range: 1.00 – 100.0 µg/L (0.0020 – 0.200mg/kg) Dried apple and tomato range: 0.500 – 50.0 µg/L (0.0020 – 0.200mg/kg)	Range: 1.00 – 100.0 µg/L (0.0020 – 0.200mg/kg) Dried apple and tomato range: 0.500 – 50.0 µg/L (0.0020 – 0.200mg/kg)	Range: 1.00 – 100.0 µg/L (0.0020 – 0.200mg/kg) Dried apple and tomato range: 0.500 – 50.0 µg/L (0.0020 – 0.200mg/kg)	Range: 1.00 – 100.0 µg/L (0.0020 – 0.200mg/kg) Dried apple and tomato range: 0.500 – 50.0 µg/L (0.0020 – 0.200mg/kg)
Assessment of matrix effects is presented	Yes The matrix effect is considered not significant according to the SANTE/2020/12830 rev.1 guideline, since the analytes responses in matrix matched calibration solutions are $< \pm 20\%$ of the analytes responses in the calibration solutions prepared in solvent (water/methanol 50:50 + 1% formic acid). Since the matrix effect is negligible, the calibration can also be done using standards prepared in solvent.			
Limit of determination/quantification	LOD: 1 µg/L LOD: 0.50 µg/L for dried apple/tomato LOQ: 0.01 mg/Kg	LOD: 1 µg/L LOD: 0.50 µg/L for dried apple/tomato LOQ: 0.01 mg/Kg	LOD: 1 µg/L LOD: 0.50 µg/L for dried apple/tomato LOQ: 0.01 mg/Kg	LOD: 1 µg/L LOD: 0.50 µg/L for dried apple/tomato LOQ: 0.01 mg/Kg

Conclusion

A mean recovery of 60-120% with a Relative Standard Deviation $\leq 30\%$ was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.1.4.2 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/09

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Dried apples

Rigamonti, E. 2022
Study No. GLP-STUDY-1088/2021

Guideline(s): Yes.
- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Postapproval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-109 (KCP 5.2.1-08) for the determination of triazole-derivative metabolites (TDMs) in dried apples in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Sample preparation

Aliquots taken from the homogenized frozen samples were weighted (about 5 g), in a 50 mL falcon plastic tube. After that 100 µL of TDM ISTD MIX and 9.0 mL of water were added. In a second time, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The separation of the organic phase was achieved by centrifugation. 1.0 mL of the supernatant was taken and filtered using PVDF syringe filter at 0.45 µm.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using fortified Dried apples samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration.

The SANTE/2020/12830 rev. 1 (2021) guideline requires any interference present in the control matrix sample(s) to be lower than 30 % of the LOQ.

No interferences above the LOD were found in the control Dried apples samples, the analysis of fortified samples at low and high level gave the following results.

Analyte	Product ion	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
1,2,4-triazole (1,2,4-TRZ)	43.1	Low	0.011	5	0.011	102.9	6.98	0.0
		High	0.106	5	0.099	97.7	1.60	0.0
1,2,4-triazole (1,2,4-TRZ)	70.0	Low	0.011	5	0.011	101.6	2.92	0.0
		High	0.106	5	0.101	99.4	2.82	0.0
Triazole alanine (TA)	70.0	Low	0.008	5	0.007	92.7	2.64	0.0
		High	0.076	5	0.076	103.6	3.14	0.0
Triazole alanine (TA)	88.0	Low	0.008	5	0.007	96.0	13.31	0.0
		High	0.076	5	0.076	104.4	2.93	0.0
Triazole lactic acid (TLA)	70.0	Low	0.010	5	0.010	103.3	2.21	0.0
		High	0.099	5	0.094	98.8	1.77	0.0
Triazole lactic acid (TLA)	43.0	Low	0.010	5	0.010	103.6	3.43	0.0
		High	0.099	5	0.093	97.9	1.51	0.0
Triazole acetic acid (TAA)	70.0	Low	0.010	5	0.011	107.9	1.27	0.0
		High	0.105	5	0.100	100.6	0.86	0.0
Triazole acetic acid (TAA)	73.0	Low	0.010	5	0.011	107.5	1.80	0.0
		High	0.0105	5	0.097	97.2	3.29	0.0

Matrix dried tomatoes	Product ion	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n=10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
1,2,4-triazole (1,2,4-TRZ)	43.1	102.9	6.98	97.7	1.60	100.3	5.61
	70.0	101.6	2.92	99.4	2.82	100.5	2.96
Triazole alanine (TA)	70.0	92.7	2.64	103.6	3.14	98.1	6.45
	88.0	96.0	13.31	104.4	2.93	100.2	9.78
Triazole lactic acid (TLA)	70.0	103.3	2.21	98.8	1.77	101.1	3.02
	43.0	103.6	3.43	97.9	1.51	100.7	3.94
Triazole acetic acid (TAA)	70.0	107.9	1.27	100.6	0.86	104.2	3.80
	73.0	107.5	1.80	97.2	3.29	102.4	5.84

Since all recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify TDMs (triazole-derivative metabolites) in Dried apples samples with an established LOQ of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC-DMS-MS/MS instrument with quantification by internal standard, was shown to be specific for TDMs (triazole-derivative metabolites) residues in Dried apples.

Matrix effect

Analyte	Precursor ion	Product Ion	m/z	Matrix effect (%)
1,2,4-triazole (1,2,4-TRZ)	70.1	Quantifier	43.1	10
Triazole alanine (TA)	157.0	Quantifier	70.0	8
Triazole lactic acid (TLA)	158.0	Quantifier	70.0	0
Triazole acetic acid (TAA)	128.0	Quantifier	70.0	0

Not significant matrix effects for all TDMs (triazole-derivative metabolites) residues in Dried apples matrix were found ($< \pm 20\%$).

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ.

The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 µg/L for each analyte, corresponding to about 0.002 mg/kg in Dried apples (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (70 – 120% with % RSD < 20%), was a final injected solution of about 2.50 µg/L for each analyte, corresponding to about 0.010 mg/kg in Dried apples

Analyte	LOD		LOQ	
	Injected concentration (µg/L)	Content in the matrix (mg/kg)	Injected concentration (µg/L)	Content in the matrix (mg/kg)
1,2,4-triazole	0.50	0.002	2.50	0.010

(1,2,4-TRZ)				
Triazole alanine (TA)	0.50	0.002	2.50	0.010
Triazole lactic acid (TLA)	0.50	0.002	2.50	0.010
Triazole acetic acid (TAA)	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Confirmation

Since the analysis by HPLC using internal standards and MS triple quadrupole detector (HPLC/MS/MS) equipped with a Differential Mobility Separation (DMS) device in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole (1,2,4-TRZ)	quantifier	70.1	43.1
	qualifier		70.0
Triazole alanine (TA)	quantifier	157.0	70.0
	qualifier		88.0
Triazole lactic acid (TLA)	quantifier	158.0	70.0
	qualifier		43.0
Triazole acetic acid (TAA)	quantifier	128.0	70.0
	qualifier		73.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in dried apples with the tested LOQ of 0.01 mg/kg.

A 2.1.1.1.4.3 Method validation

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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Reference: KCP 5.1.2/10

Report Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Dried tomatoes
 Rigamonti, E. 2022
 Study No. GLP-STUDY-1089/2021

Guideline(s): Yes.
 - European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.1 (24/02/2021).

Deviations: No

GLP: Yes
Acceptability: Yes

Materials and methods

The objective of the study was to independently validate an analytical method as validated by study number GLP-STUDY-21-109 (KCP 5.1.2-08) for the determination of TDMs (triazole-derivative metabolites) in dried tomatoes in accordance to the guidance document SANTE/2020/12830, rev. 1 with a limit of quantification of 0.01 mg/kg.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Both repeatability and recovery tests were performed using fortified Dried tomatoes samples, that were fortified five times at about 0.010 mg/kg (LOQ) and five times at 0.100 mg/kg (10 x LOQ), as nominal concentration.

Since no matrix sample (dried tomatoes) with interference lower than 30% of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine and the Triazole-lactic acid analytes was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.

Analyte	Production	Level	Spike (mg/kg)	Tests No.	Mean (mg/kg)	Mean recovery (%)	RSD%	Interference (%)
1,2,4-triazole (1,2,4-TRZ)	43.1	Low	0.011	5	0.010	99.3	9.66	0.0
		High	0.105	5	0.109	105.5	2.50	0.0
1,2,4-triazole (1,2,4-TRZ)	70.0	Low	0.011	5	0.010	100.1	5.85	0.0
		High	0.105	5	0.112	108.1	1.20	0.0
Triazole-alanine (TA)	70.0	Low	0.008	5	0.007	97.0	3.55	70.4
		High	0.078	5	0.064	84.0	4.69	25.0
Triazole-alanine (TA)	88.0	Low	0.008	5	0.008	100.3	5.73	67.0
		High	0.078	5	0.069	90.2	8.62	22.1
Triazole-lactic acid (TLA)	70.0	Low	0.010	5	0.010	101.9	1.99	33.9
		High	0.098	5	0.105	108.4	0.46	4.6
Triazole-lactic acid (TLA)	43.0	Low	0.010	5	0.009	97.9	7.72	32.1
		High	0.098	5	0.104	107.7	0.24	4.3
Triazole-acetic acid (TAA)	70.0	Low	0.010	5	0.010	100.5	1.61	0.0
		High	0.103	5	0.109	108.0	0.55	0.0
Triazole-acetic acid (TAA)	73.0	Low	0.010	5	0.010	103.1	7.40	0.0
		High	0.103	5	0.110	108.2	0.95	0.0

Matrix Dried to-matoes	Production	Spike Low or LOQ (n = 5)		Spike High or 10 x LOQ (n = 5)		Overall (n = 10)	
		Mean	RSD%	Mean	RSD%	Mean	RSD%
	43.1	99.3	9.66	105.5	2.50	102.4	7.22

1,2,4-triazole (1,2,4-TRZ)	70.0	100.1	5.85	108.1	1.20	104.1	5.59
Triazole-alanine (TA)	70.0	97.0	3.55	84.0	4.69	90.5	8.49
	88.0	100.3	5.73	90.2	8.62	95.3	8.79
Triazole-lactic acid (TLA)	70.0	101.9	1.99	108.4	0.46	105.2	3.52
	43.0	97.9	7.72	107.7	0.24	102.8	7.03
Triazole-acetic acid (TAA)	70.0	100.5	1.61	108.0	0.55	104.3	3.93
	73.0	103.1	7.40	108.2	0.95	105.6	5.49

According to the SANTE/2020/12830 rev. 1 guideline's requirement, the mean recovery values must be: in the range 60 to 120 % for concentration level ≤ 0.01 mg/kg, with an RSD% lower than 30%, in the range 70 to 120 % for concentration level $> 0.01 - \leq 0.1$ mg/kg, with an RSD% lower than 20%. Since all recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the correct range, these criteria were fulfilled and therefore the analytical method can be considered suitable to quantify TDMs (triazole-derivative metabolites) in dried tomatoes samples with an established LOQ of 0.010 mg/kg.

Specificity (Selectivity)

The analytical method, using the HPLC-DMS-MS/MS instrument with quantification by internal standard, was shown to be specific for TDMs (triazole-derivative metabolites) residues in dried tomatoes.

Matrix effect

Analyte	Precursor ion (m/z)	Product Ion	m/z	Matrix effect (%)
1,2,4-triazole (1,2,4-TRZ)	70.1	Quantifier	43.1	6
Triazole alanine (TA)	157.0	Quantifier	70.0	-17
Triazole lactic acid (TLA)	158.0	Quantifier	70.0	3
Triazole acetic acid (TAA)	128.0	Quantifier	70.0	-3

Not significant matrix effects for all TDMs (triazole-derivative metabolites) residues in Dried tomatoes matrix were found ($< \pm 20\%$).

Therefore, the calibration standards could be prepared in solvent or in matrix.

In this case, since no matrix sample free from the Triazole-alanine and the Triazole-lactic acid analytes were available and the matrix effect was negligible, it was necessary to prepare the calibration standard solution in solvent.

Limit of detection (LOD) and Limit of quantification (LOQ)

Data from Linearity test were used to calculate the LOD whereas data from Recovery test were used to calculate the LOQ.

The limit of detection (LOD), defined as the lowest working standard solution WSS 1, was a final injected solution of about 0.50 $\mu\text{g/L}$ for each analyte, corresponding to about 0.002 mg/kg in Dried tomatoes (30% of the LOQ).

The limit of quantification (LOQ), defined as the lowest fortification level with acceptable recovery and repeatability (60 – 120% with % RSD $< 30\%$), was a final injected solution of about 2.50 $\mu\text{g/L}$ for each

analyte, corresponding to about 0.010 mg/kg in Dried tomatoes.

Analyte	LOD		LOQ	
	injected concentration (µg/L)	Nominal content in the matrix (mg/kg)	Nominal injected concentration (µg/L)	Nominal content in the matrix (mg/kg)
1,2,4-triazole (1,2,4-TRZ)	0.50	0.002	2.50	0.010
Triazole-alanine (TA)	0.50	0.002	2.50	0.010
Triazole-lactic acid (TLA)	0.50	0.002	2.50	0.010
Triazole-acetic acid (TAA)	0.50	0.002	2.50	0.010

The LOD and the LOQ was successfully established for each analyte and both mass transitions.

Analyte results calculated as < 0.002 mg/kg (LOD) are classified as not detectable (n.d.).

Analyte results calculated as greater than the limit of detection but less than the limit of quantification, are designated as < 0.010 mg/kg.

If the analyte content is calculated as greater than 0.200 mg/kg in Dried tomatoes, the final solution must be suitably diluted using volumetric glassware to fit in the calibration range.

Confirmation

Since the analysis by HPLC using internal standards and MS triple quadrupole detector (HPLC/MS/MS) equipped with a Differential Mobility Separation (DMS) device in MRM mode is highly specific and gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.

The selectivity of the primary method was demonstrated with the obtained data from the qualifier transitions.

Analyte	Transition type	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole (1,2,4-TRZ)	quantifier	70.1	43.1
	qualifier		70.0
Triazole alanine (TA)	quantifier	157.0	70.0
	qualifier		88.0
Triazole lactic acid (TLA)	quantifier	158.0	70.0
	qualifier		43.0
Triazole acetic acid (TAA)	quantifier	128.0	70.0
	qualifier		73.0

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1, for the determination of TDMs (triazole-derivative metabolites) in dried tomatoes with the tested LOQ of 0.01 mg/kg.

A 2.1.1.2 Description of analytical methods for the determination of residues in animal matrices il (KCP 5.2)

Comments of zRMS:	-Method is acceptable
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A 2.1.1.2.1 Analytical method 1 (Honey)

A 2.1.1.2.1.1 Method Validation 1

A 2.1.1.2.1.2

Reference:	KCP 5.2/01
Report	Analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivative Metabolites (TDMs) residue in Honey Ivo Rovetto. 2023 Study code. 1111.4F.SAG23
Guidelines:	Yes. <ul style="list-style-type: none">• European Community Guidelines 7029/VI/95 – Rev. 5, 22/07/97: General recommendations for the design, preparation and realization of residue trials.• Regulation (EC) no. 1107/2009 of the European Parliament and of the Council of 21st October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EC and 91/414/EEC, 21/10/2019• Regulation (EU) No. 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, 01/03/2013• OECD Test Guideline 509: Crop field trial• European Community Guidelines SANTE/2019/12752 on data requirements for setting maximum residue levels, comparability of residue trials and extrapolation of residue data on products from plant and animal origin (Repealing and replacing the existing Guidance Document SANCO 7525/VI/95 Rev. 10.3)• SANTE/11956/2016 rev. 9, Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey, 14 September 2018.• SANTE/2020/12830, Rev.2 14 February 2023, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.• Guidance document on pesticide residue analytical methods [ENV/JM/MONO(2007)17].• GLP compliance monitoring of international multi-site studies, Ref. Ares(2017)75658.• Directive 2004/10/EC of the European Parliament and of the Council (L 50/44).
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Principle of the test

The validation of the analytical method was carried out under GLP compliance to SANTE/11956/2016 rev. 9 (Appendix V: tunnel residue trials for MRL setting in Honey). The analyses were carried out according to the analytical method for the determination of difenoconazole, prothioconazole and prothioconazole-

desthio and according to the analytical method for the determination of TDMs (triazole-derivative metabolites) validated under GLP compliance according to SANTE/12830/2020 rev. 2 guideline.

Description of the method

Determination of difenoconazole, prothioconazole and prothioconazole-desthio:

The analytical method to quantify Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey was based on the QuEChERS method (EN 15662_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry).

Sample preparation:

Aliquots of 5 g of specimen were taken from the frozen samples and put in a 50 mL screw capped centrifuge PE test tube (aliquots for recovery evaluation were spiked at this stage), followed by the addition of 5 mL of an aqueous solution of 0.1 g/L of cysteine hydrochloride (to prevent the degradation of Prothioconazole) and 0.1% formic acid.

Then, 20 mL of acetonitrile were added and the obtained mixture was vigorously shaken for one minute. After that, a packet of unbuffered QuEChERS extraction salt (4.0 g MgSO₄, 1.0 g NaCl) was added and the mixture shaking again. The separation of the organic phase was achieved by centrifugation at 5000 rpm for 5 minutes. Finally, an aliquot of the organic supernatant was filtered, transferred in a 2 mL HPLC glass vial and analysed with a HPLC-MS/MS system.

Reference solutions preparation:

The following reference solutions were prepared:

Solution	Starting material	Weight /Volume	Final volume (mL) (acetonitrile)	Analyte	Actual Conc. (mg/L)*	Preparation date (dd/mm/yyyy)	Expiry date (dd/mm/yyyy)**
Difenoconazole stock solution (Difenoconazole ~ 1000 mg/L)	Difenoconazole reference material (purity: 95.4%)	8.52 mg	10	Difenoconazole	813	14/09/2023	19/12/2023
Prothioconazole stock solution (Prothioconazole ~ 1000 mg/L)	Prothioconazole Reference material (purity: 98.2 %)	6.63 mg	10	Prothioconazole	651	14/09/2023	08/11/2023
Prothioconazole-desthio stock solution (Prothioconazole- desthio ~ 1000 mg/L)	Prothioconazole-desthio Reference material (purity: 98.3 %)	5.75 mg	10	Prothioconazoledesthio	565	14/09/2023	19/12/2023
Solution A (~ 10 mg/L each analyte)	Difenoconazole stock solution	123 µL	10	Difenoconazole	10.0	Each Analytical Session	Daily
	Prothioconazole stock solution	154 µL		Prothioconazole	10.0		
	Prothioconazole- desthio stock solution	177 µL		Prothioconazoledesthio	10.0		
Solution B (~ 1 mg/L each analyte)	Solution A	1000 µL	10	Difenoconazole	1.00		
				Prothioconazole	1.00		
				Prothioconazoledesthio	1.00		

* Values corrected for the purity

** For stability data of stock solutions refer to the validation study LBN-0092-2023 (stability of 96 days from the preparation for Difenoconazole and Prothioconazole-desthio, 55 days for Prothioconazole)

Matrix-matched standard solutions:

matrix-matched analytical standard solutions were prepared at each analytical session from Solution B and A using the final extract of an unfortified sample extract on the basis of the following scheme:

Matrix-matched standard solutions					
Solution	µL of solution		Final volume (mL)	Concentration in solution (each analyte) (µg/L)	Nominal concentration on sample (each analyte) ¹ (mg/kg)
L1	1.0	Solution B	2	0.500	0.0020
L2	2.5	Solution B	1	2.50	0.0100
L3	5.0	Solution B	1	5.00	0.020
L4	2.5	Solution A	1	25.0	0.100
L5	5.0	Solution A	1	50.0	0.200

¹ Calculated considering the nominal sample preparation (5 g to a final volume of 20 mL)

Instrumental determination

The analysis of Difenoconazole, Prothioconazole and Prothioconazole-desthio was carried out using a HPLCMS/MS system operating in the following conditions:

Instrument: HPLC Agilent 1290 Infinity II coupled with a triple quadrupole mass spectrometer Agilent 6470A

Column: Phenomenex Kinetex C18, 1.7 µm, 2.1 x 50 mm

Column temperature: 40°C

Flow: 0.6 mL/min

Injection volume: 2.5 µL

Mobile phase A: LC-MS grade water with 0.2 % formic acid and 5 mM ammonium formate - Mobile
phase B: LC-MS grade methanol with 0.2 % formic acid and 5 mM ammonium formate - Elution:
gradient of the following composition:

Time (min)	% A	% B
0	70	30
0.5	70	30
3.0	0	100

Stop time: 5 min

Post time: 1 min

Divert valve: 0 min. to waste, 2 min. to MS, 3.5 min. to waste

Source type: ESI

Gas temperature: 350°C

Gas flow (L/min): 5

Nebulizer (psi): 40

Sheath gas heater: 400°C

Sheath gas flow (L/min): 12

Capillary: positive mode 3500 V, negative mode 3000 V

Vcharging: 0

Acquiring mode: ESI positive and ESI negative, MRM (multi-reaction monitoring).

MRM monitored transitions								
Analyte	Retention time (approx, min)	Detection	Polarity	Precursor ion (m/z)	Product ion (m/z)	Dwell (mS)	Fragmentor (V)	Collision energy (eV)
Difenoconazole	2.9	Primary	Positive	406.2	251.1	50	115	22
		Confirmatory		406.2	188.4	50		54
Prothioconazole-desthio	2.6	Primary	Positive	312.2	125	50	100	38
		Confirmatory		312.2	69.8	50		26
Prothioconazole	2.8	Primary	Negative	342	100	50	115	25
		Confirmatory		342	264	50		21

Calibration:

The quantification of each analyte was made through the building of a calibration straight line with the external standard method. 5 matrix-matched analytical standard solutions were analysed in single injections in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy)

During the analytical session for the determination of Difenoconazole, Prothioconazole and Prothioconazole-desthio, two recovery check tests were carried out fortifying aliquots of an untreated honey sample (CDS23-1469 and CDS-23-1565) at the following fortification levels:

- LOQ (0.01 mg/kg), adding 50 µL of Solution B (containing 1.00 mg/L of each analyte) to aliquots of about 5 g of untreated sample.
- 10xLOQ (0.1 mg/kg), adding 50 µL of Solution A (containing 10.0 mg/L of each analyte) to aliquots of about 5 g of untreated sample.

Procedural recoveries were handled and stored in the same way and for the same time period as the analytical specimens that have been prepared within the same analytical set. Procedural recovery values were evaluated against the SANTE 2020/12830 rev.2 requirements

Determination of TDM

Principle of the test:

The applied analytical method (AM-LBN-0093-2023) allows the determination of the following TDM (triazole derivative metabolites):

- 1,2,4-triazole (TRZ)
- Triazole-alanine (TA)
- Triazole-lactic acid (TLA)
- Triazole-acetic acid (TAA) in honey.

The analytical method was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPE-PO-Method) - Method 8 (M8)”. The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).

Sample preparation:

An aliquot of about 5 g of honey was taken from the frozen sample and put in a 50 mL screw capped centrifuge PE test tube (aliquots for recovery evaluation were spiked at this stage) followed by the addition of 100 µL of the internal standard solution TDM ISTD MIX (2 mg/L of each internal standard) and of 7.5 mL of deionized water. After a vigorous shake, 10 mL of 1% formic acid in methanol were added and the obtained mixture was shaken for about one minute. An aliquot of about 1 mL the supernatant was taken, filtered with a 0.45 µm PVDF filter and transferred in a 2 mL HPLC glass vial for the final analysis with a HPLC-DMS-MS/MS system.

Stock standard

Solution	Starting material	Weight (mg)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)*	Preparation date (dd/mm/ yyyy)	Expiry date (dd/mm/ yyyy)***
1,2,4-TRZ stock solution	1,2,4-triazole reference material (purity: 99.7%)	10.05	10	1,2,4-triazole	1002	14/09/2023	01/12/2023
TA stock solution	Triazole-alanine reference material (purity: 98.3%)	10.63	10	Triazole-alanine	1045	14/09/2023	01/12/2023
TLA stock solution	Triazole-lactic acid hydrochloride reference material (purity: 78.5%)	11.73	10	Triazole-lactic acid	747.4**	14/09/2023	01/12/2023
TAA stock solution	Triazole-acetic acid reference material (purity: 97%)	10.75	10	Triazole-acetic acid	1043	14/09/2023	01/12/2023

Internal standard stock solutions

Solution	Starting material	Weight (mg)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)*	Preparation date (dd/mm/ yyyy)	Expiry date (dd/mm/ yyyy)**
TRZ ISTD stock solution	1,2,4-triazole[¹³ C ₂ , ¹⁵ N ₃] reference material (purity: 98.4%)	1.31	1	1,2,4-triazole[¹³ C ₂ , ¹⁵ N ₃]	1289	14/09/2023	01/12/2023
TA ISTD stock solution	Triazole-alanine D2 homogeneous solution in water/methanol 3:1 v/v	/	/	Triazole-alanine D2	999.68	/	14/08/2024
TLA ISTD stock solution	Triazole[¹³ C ₂ , ¹⁵ N ₃] lactic acid reference material (purity: 98.42%)	1.46	1	Triazole[¹³ C ₂ , ¹⁵ N ₃] lactic acid	1437	14/09/2023	01/12/2023
TAA ISTD stock solution	Triazole[¹³ C ₂ , ¹⁵ N ₃] acetic acid reference material (purity: 98.03%)	1.04	1	Triazole[¹³ C ₂ , ¹⁵ N ₃] acetic acid	1020	14/09/2023	01/12/2023

Diluted solutions

Solution	Starting material	Volume (μL)	Final volume (mL) (water)	Analyte	Actual concentration (mg/L)	Preparation date (dd/mm/ yyyy)	Expiry date (dd/mm/ yyyy)**
TDM Mix Solution A	1,2,4-TRZ stock solution	100	10	1,2,4-triazole	10.0	Each Analytical Session	Daily
	TA stock solution	96		Triazole-alanine	10.0		
	TLA stock solution	134		Triazole-lactic acid	10.0		
	TAA stock solution	96		Triazole-acetic acid	10.0		
TDM Mix Solution B	TDM Mix Solution A	1000	10	1,2,4-triazole	1.00		
				Triazole-alanine	1.00		
				Triazole-lactic acid	1.00		
				Triazole-acetic acid	1.00		
TDM ISTD MIX	TRZ ISTD stock solution	18.1	10	1,2,4-triazole[¹³ C ₂ , ¹⁵ N ₃]	2.00		
	TA ISTD stock solution	20		Triazole-alanine D2	2.00		
	TLA ISTD stock solution	13.7		Triazole[¹³ C ₂ , ¹⁵ N ₃] lactic acid	2.00		
	TAA ISTD stock solution	16.3		Triazole[¹³ C ₂ , ¹⁵ N ₃] acetic acid	2.00		

Working standard solutions

Working standard solutions						
Solution	μL of TDM Mix Solution B	μL of TDM ISTD MIX	Final volume (mL)	TDM concentration (each analyte) (μg/L)	ISTD concentration (each compound) (μg/L)	Nominal concentration on the sample ¹ (mg/kg)
L1	1	10	2	0.5	10	0.002
L2	2.5	5	1	2.5	10	0.010
L3	10	5	1	10	10	0.040
L4	25	5	1	25	10	0.100

L5	50	5	1	50	10	0.200
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Instrumental conditions

The analyses were carried out using a HPLC-DMS-MS/MS system according to the following conditions:

Instrument: HPLC Shimadzu LC-40 XR + Sciex API 6500 + equipped with SelexION+ (Differential Ion Mobility Device)

Column: Thermo Hypercarb 5 µm, 2.1 x 100 mm

Column temperature: 40°C

Flow: 0.6 mL/min

Injection volume: 2 µL

Mobile phase A: LC-MS grade water with 1 % acetic acid - Mobile phase B: LC-MS grade methanol with 1 % acetic acid - Elution: gradient of the following composition:

Time (min)	% A	% B
0	100	0
6.00	10	90
7.00	10	90
7.10	100	0

Stop time: 10 min

Source type: ESI

Curtain gas flow: 30 mL/min

Gas 1: 55 mL/min

Gas 2: 65 mL/min

Gas temperature: 500°C

Capillary: positive mode 3500 V

Acquiring mode: ESI positive, MRM (multi-reaction monitoring).

Transitions: the optimised MS/MS fragmentation parameters are reported in the table below:

MRM monitored transitions:

Compound	Rt (min)	Detection	Associated internal standard	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole (1,2,4-TRZ)	1.0	Primary	TRZ ISTD	70.1	43.1
		Confirmatory	TRZ ISTD	70.1	70.0
Triazole alanine (TA)	1.05	Primary	TAL ISTD	157.0	70.0
		Confirmatory	TAL ISTD	157.0	88.0
Triazole lactic acid (TLA)	3.4	Primary	TLA ISTD	158.0	70.0
		Confirmatory	TLA ISTD	158.0	43.0
Triazole acetic acid (TAA)	3.8	Primary	TAA ISTD	128.0	70.0
		Confirmatory	TAA ISTD	128.0	73.0
1,2,4-Triazole- [13C2,15N3]	1.0	Primary	-	75.0	46.0
1,2,4-Triazole Alanine [D2]	1.05	Primary	-	159.0	72.0
1,2,4-Triazole- [13C2, 15N3] Lactic Acid	3.4	Primary	-	163.0	75.0
1,2,4-Triazole acetic acid [13C2, 15N3]	3.8	Primary	-	133.0	75.0

Calibration

The quantification of each analyte was made through the building of a calibration straight line with the internal standard method. 5 analytical standard solutions were analysed in single injections in order to obtain a calibration curve (1/x weighed) interpolated with a linear regression.

Recovery (Accuracy)

During the analytical session for the determination of TDM (1,2,4-triazole, triazole-alanine, triazole-lactic acid and triazole-acetic acid), two recovery check tests were carried out fortifying aliquots of an untreated honey sample (CDS-23-1469 and CDS-23-1565) at the following fortification levels:

- LOQ (0.01 mg/kg), adding 50 µL of TDM Mix solution B (containing 1.00 mg/L of each TDM) to aliquots of about 5 g of untreated sample.
- 10xLOQ (0.1 mg/kg), adding 50 µL of TDM Mix solution A (containing 10.0 mg/L of each TDM) to aliquots of about 5 g of untreated sample.

Results and discussions

Method validation data can be summarised in the tables below.

Table A 6: Recovery results from method validation of Difenoconazole using the analytical method

Difenoconazole - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.0100	2.6912	0.0108	108
CDS-23-1469 RC2	10xLOQ	0.100	29.2820	0.117	117
CDS-23-1565 RC1	LOQ	0.00988	2.3309	0.00921	93.2
CDS-23-1565 RC2	10xLOQ	0.0998	24.9968	0.0998	100

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

Prothioconazole - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.0100	1.9831	0.00793	79.3
CDS-23-1469 RC2	10xLOQ	0.100	17.5604	0.0702	70.2
CDS-23-1565 RC1	LOQ	0.00988	2.4439	0.00966	97.8
CDS-23-1565 RC2	10xLOQ	0.0998	26.9483	0.108	108

Table A 8: Recovery results from method validation of Prothioconazole-desthio using the analytical method

Prothioconazole-desthio - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.0100	2.4187	0.00967	96.7
CDS-23-1469 RC2	10xLOQ	0.100	27.1363	0.109	109
CDS-23-1565 RC1	LOQ	0.00988	2.3981	0.00948	95.9
CDS-23-1565 RC2	10xLOQ	0.0998	25.2165	0.101	101

Table A 10: Characteristics for the analytical method used for validation of Difenoconazole, Prothioconazole, Prothioconazole-desthio residues in Honey

	Difenoconazole	Prothioconazole	Prothioconazole-desthio
Calibration (range, number of data points)	Exp. Range = 0.5000-50.0000 $R^2 = 0.99988849$ $y = 1578.707297x + 326.624111$ (weighting: 1/x)	Exp. Range = 0.5000-50.0000 $R^2 = 0.99826763$ $y = 18.433477x - 1.901495$ (weighting: 1/x)	Exp. Range = 0.5000-50.0000 (n=5) $R^2 = 0.99971858$ $Y = 517.190437x + 274.256963$ (weight: 1/x)
Limit of determination/quantification	LOD: 0.002 mg/kg LOQ: 0.010 mg/kg		

Table A 9: Recovery results from method validation of 1,2,4-triazole (1,2,4-TRZ) using the analytical method

1,2,4-triazole (TRZ) - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.00988	2.342	0.00926	93.7
CDS-23-1469 RC2	10xLOQ	0.0990	23.924	0.0947	95.7
CDS-23-1565 RC1	LOQ	0.0101	2.022	0.00817	80.9
CDS-23-1565 RC2	10xLOQ	0.0994	24.699	0.0982	98.8

Table A 10: Recovery results from method validation of Triazole-alanine (TA) using the analytical method

Triazole alanine (TA) - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.00988	2.470	0.00976	98.8
CDS-23-1469 RC2	10xLOQ	0.0990	21.860	0.0866	87.4
CDS-23-1565 RC1	LOQ	0.0101	1.929	0.00779	77.2
CDS-23-1565 RC2	10xLOQ	0.0994	21.878	0.0870	87.5

Table A 11: Recovery results from method validation of Triazole-lactic acid (TLA) using the analytical method

Triazole lactic acid (TLA) - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.00988	2.361	0.00933	94.4
CDS-23-1469 RC2	10xLOQ	0.0990	25.383	0.101	102
CDS-23-1565 RC1	LOQ	0.0101	1.970	0.00796	78.8
CDS-23-1565 RC2	10xLOQ	0.0994	25.164	0.100	101

Table A 12: Recovery results from method validation of Triazole-acetic acid (TAA) using the analytical method

Triazole acetic acid (TAA) - Procedural recovery check results					
Specimen identification	Fortification level	Actual spiked Conc. (mg/kg)	Measured conc. in the analyzed extract (µg/L)	Measured conc. in the sample (mg/kg)	Recovery (%)
CDS-23-1469 RC1	LOQ	0.00988	2.403	0.00950	96.1
CDS-23-1469 RC2	10xLOQ	0.0990	25.193	0.0998	101
CDS-23-1565 RC1	LOQ	0.0101	2.571	0.0104	103
CDS-23-1565 RC2	10xLOQ	0.0994	25.314	0.101	101

Table A 10: Characteristics for the analytical method used for validation of TDMs residues in Honey

	1,2,4-triazole (1,2,4-TRZ)	Triazole alanine (TA)	Triazole lactic acid (TLA)	Triazole acetic acid (TAA)
Calibration (range, number of data points)	Range = 0.511-50.169 (n=5) $R^2 = 0.9984805776$ $y = 0.64316x + 0.00558$ (r = 0.99924) (weighting: 1/x)	Range = 0.60-51.869 (n=5) $R^2 = 0.9950661009$ $y = 14.22980x + 0.04992$ (r = 0.99753) (weighting: 1/x)	Range = 0.513-50.20 (n=5) $R^2 = 0.9989602704$ $y = 0.17746x + 0.00104$ (r = 0.99948) (weighting: 1/x)	Range = 0.543-50.748 (n=5) $R^2 = 0.9993601024$ $y = 1.06430x + 0.00787$ (r = 0.99968) (weighting: 1/x)
Limit of determination/quantification	LOD: 0.002 mg/kg LOQ: 0.010 mg/kg			

Conclusion

The test item was applied, and specimens were generated and analysed according to the recommendations of the sponsor and the study objectives. Results of residue analysis may therefore be used in order to predict the residue behaviour of Difenoconazole, Prothioconazole and Prothioconazole-desthio after one foliar application of the test item IN233C1560 380 EC when applied as per study indications.

zRMS

Reporting Table – replay to DE comment: “The method of Rovetto (2023) is a method for risk assessment and should be deleted from the section for monitoring methods. With only two fortifications per level the method does not meet the validation criteria for new risk assessment methods according to SANTE/2020/12830 rev. 2 and should be considered not acceptable.”

The analytical method of Rovetto (2023) was validated under GLP compliance according to SANTE/2020/12830 Rev.2 in studies LBN-0092-2023 (Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey) and LBN-0093-2023 (Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey).

Report	Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey. Longhi, D. 2023 Report No LBN-0092-2023
Guidelines:	Yes. European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev. 2 (14/02/2023) - Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residues Levels in honey, SANTE/11956/2016 rev. 9, 14 September 2018

- Guidance document on pesticide residue analytical methods [ENV/JM/MONO(2007)17] (13/08/2007).
- European Commission (2017): SANTE/2017/10632 Rev. 5 (11 May 2023): Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods
- European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method
- CIPAC MT 75.3 "Determination of pH values".
- GLP-STUDY-21-31 "Validation of an analytical method for the quantification of Difenconazole and Prothioconazole-desithio in wheat, barley, oilseed rape and processed commodities", Test Facility: LabAnalysis, Study Director: Diego Longhi

Deviations: No

GLP: Yes

Acceptability: Yes

The analytical method (Report No LBN-0092-2023) was based on the method "European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method".

The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).

The limit of quantification (LOQ) was set to 0.01 mg/kg.

Report Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey. Longhi, D. 2023.

Guidelines: Yes.
European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.2 (14/02/2023).
- European Commission (2017): SANTE 2017/10632 rev. 5, dated 11 May 2023: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.
- OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17.
- Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. Food of Plant Origin (QuPPe-PO-Method) Version 12.1 (17.03.2023).
- CIPAC MT 75.3 "Determination of pH values".
- GLP-STUDY-21-108 "Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities", test facility: LabAnalysis srl, study director: Diego Longhi

Deviations: No

GLP: Yes

Acceptability: Yes

The analytical method (Report No LBN-0092-2023) was based on the method "Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)".

The analytical method was based on an extraction with a mixture of water/methanol with 1% of formic acid and on an instrumental determination using a HPLC-DMS-MS/MS (high-performance liquid chromatography + Differential ion mobility-triple-quadrupole mass spectrometry).

The limit of quantification (LOQ) was set to 0.01 mg/kg.

In study report **LBN-0092-2023**, five fortified replicates were performed per level for Difenoconazole, Prothioconazole and Prothioconazole-desithio. The mean recoveries per level found for both primary and confirmatory transitions were in compliance with the requirements set in SANTE/2020/12830 rev. 2:

- LOQ level (0.01 mg/kg): recoveries range of 60- 120% and RSD \leq 30%
- 10xLOQ level (0.1 mg/kg): recoveries range 70- 120% and RSD \leq 20%

Table 1. Summary of recovery and repeatability for difenoconazole – see below Reporting table

Matrix	Level (mg/kg)	Replicates	Primary transition (406.2/251.1)			Confirmatory transition (406.2/188.4)		
			Mean % recovery	SD	%RSD	Mean % recovery	SD	%RSD
Honey	0.01 (LOQ)	5	98.7	5.7	5.7	99.3	4.1	4.1
	0.1 (10xLOQ)	5	100	2.8	2.8	98.2	3.9	4.0
	Overall	10	99.1	4.2	4.3	98.7	3.8	3.9

In study report **LBN-0093-2023**, five fortified replicates were performed per level for 1,2,4-triazole (1,2,4-TRZ), triazole-alanine (TA), triazole-lactic acid (TLA), triazole-acetic acid (TAA). The mean recoveries per level found for both primary and confirmatory transitions were in compliance with the requirements set in SANTE/2020/12830 rev. 2:

- LOQ level (0.01 mg/kg): recoveries range of 60- 120% and RSD \leq 30%
- 10xLOQ level (0.1 mg/kg): recoveries range 70- 120% and RSD \leq 20%

Table 2. Summary of recovery and repeatability for 1,2,4-TRZ – see below Reporting table

Matrix	Level (mg/kg)	Replicates	Primary transition (70.1/43.1)			Confirmatory transition (70.1/70)		
			Mean % recovery	SD	%RSD	Mean % recovery	SD	%RSD
Honey	0.01 (LOQ)	5	99.6	3.5	3.6	101	3.2	3.1
	0.1 (10xLOQ)	5	103	1.5	1.5	102	3.3	3.3
	Overall	10	101	3.1	3.1	101	3.1	3.0

Table 3. Summary of recovery and repeatability for TA – see below Reporting table

Matrix	Level (mg/kg)	Replicates	Primary transition 157/70			Confirmatory transition 157/88		
			Mean % recovery	SD	%RSD	Mean % recovery	SD	%RSD
Honey	0.01 (LOQ)	5	103	8.0	7.7	103	4.8	4.7
	0.1 (10xLOQ)	5	96.9	3.3	3.4	98.1	3.0	3.1
	Overall	10	100	6.7	6.7	101	4.6	4.6

Table 4. Summary of recovery and repeatability for TLA – see below Reporting table

Matrix	Level (mg/kg)	Replicates	Primary transition 158/70			Confirmatory transition 158/43		
			Mean % recovery	SD	%RSD	Mean % recovery	SD	%RSD
Honey	0.01 (LOQ)	5	106	3.1	2.9	107	5.1	4.8
	0.1 (10xLOQ)	5	105	2.9	2.8	103	2.4	2.3
	Overall	10	105	2.9	2.7	105	4.2	4.0

Table 5. Summary of recovery and repeatability for TAA – see below Reporting table

Matrix	Level (mg/kg)	Replicates	Primary transition 128/70			Confirmatory transition 128/73		
			Mean % recovery	SD	%RSD	Mean % recovery	SD	%RSD
Honey	0.01 (LOQ)	5	104	3.7	3.5	116	5.0	4.4
	0.1 (10xLOQ)	5	98.0	1.6	1.6	98.7	2.4	2.4
	Overall	10	101	4.1	4.1	107	9.9	9.2

LBN-0092-2023 and LBN-0093-2023 Reports are available upon request.

A 2.1.1.2.2 Independent Laboratory Validation (ILV) Analytical method 2

A 2.1.1.2.2.1 Method validation

Comments of zRMS:	Method is acceptable
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RMS: A statement justifying difenoconazole peak is the blank sample and in the solvent wash is provided from the laboratory.

Statement, Mattioli, B. (2024)

Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivative Metabolites (TDMs) residue in Honey

Study No. CH – 0859-2023

Lab Analysis Life Science s.r.l.

“An Interference was detected in the system, which, despite numerous attempts to clean the instrument (e.g., replacing tubes, changing several batches of solvents used as eluents, cleaning specific glassware as nebulizer or metallic part as the cones), could not be avoided.

With the injection of an empty vial, so injecting just air in the system, it was confirmed the interference, and it was concluded that the system needed an internal maintenance, with no possibility for the lab technician to fix the problem and no way for the Agilent technician to schedule an assistance intervention in time before the deadline for the final report.

In the SANTE/2020/12830. Rev 2, it is not detailed or made explicit if the interference, that has to be “less than 30% of the LOQ”, is related to the area signal or to the concentration.

In this GLP study it was considered as the concentration found in the wash and in the control sample.

Considering the obtained results in the GLP study CH-0859-2023, it can be concluded that:

- the LOD of the method (0.002 mg/kg, corresponds to the WSS 1) is less than 30% of the LOQ (0.01 mg/kg);*
- the wash and control sample have a concentration lower than LOD.*

For this reason, it is possible to set that the wash and the control sample are themselves less than 30% of the LOQ and therefore they are in specification with the SANTE/2020/12830. Rev 2.”

RMS agrees with the applicant.

Reference:

KCP 5.2/02

Report	Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivative Metabolites (TDMs) residue in Honey Mattioli B. 2023 GLP Study No. CH – 0859-2023
Guideline(s):	Yes. <ul style="list-style-type: none"> - Council Decision [C(97)186/Final] amending Annex II to the council decision concerning the mutual acceptance of data in the assessment of chemicals [C(81) 30 (final)]. - ENV/MC/CHEM(98)17 OECD Principles on Good Laboratory Practice, No. 1 and all subsequent OECD consensus documents. - Directive 2004/9/CE and directive 2004/10/CE of the European Parliament and of the Council of February 11th, 2004 enforced by Italian Legislative Decree No. 50 of March 2nd, 2007 as published in G.U. No. 86 of April 13th, 2007.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Description of the method for the quantification of Prothioconazole, Prothioconazole-desthio and difenoconazole

Recovery and Repeatability (Precision)

The recovery and the repeatability (as precision, % RSD) of the analytical method were determined using freshly fortified control samples of honey. Since the validation of the linearity and calibration test was not a requirement, but the calibration is necessary to quantify the fortified matrix samples, it is presented below how the calibration was prepared and the obtained results. Fortified samples were quantified using the equation of the calibration curve, with the standard solutions injected in the same run with the samples: five matrix-matched standard solutions (from MSS1 to MSS5) were prepared in matrix (blank honey extract) and they were injected before the fortified samples. Fortification levels were chosen at LOQ 0.01 mg/kg and at 10xLOQ 0.1 mg/kg in honey, as nominal concentrations.

Sample extraction

Reference solutions preparation:

The following reference solutions were prepared:

Instrument conditions:

The analyses were carried out using a HPLC-MS/MS system according to the following conditions:

Method for the quantification of Prothioconazole, Prothioconazole-desthio and difenoconazole			
Column	HPLC column, Kinetex C18 100 Å, 1.7 µm, 50 x 2.1 mm i.d. (+ pre-column); Internal code LCN 431 (or equivalent)		
Detector	MS Triple quadrupole (Scan in MRM mode)		
Eluent C	Methanol with formic acid 0.2% and ammonium formate 5 mM		
Eluent D	Water with formic acid 0.2% and ammonium formate 5 mM		
Eluent flow	0.6 mL/min		
Elution mode	gradient condition		
Solvent composition	Time (min)	% C	% D

	0	30	70
	0.5	30	70
	3	10	100
	5	30	70
Column temperature	40°C		
Volume of injection	2.5 µL		
Retention time	Prothioconazole - About 3.2 minutes		
	Prothioconazole-desthio - About 3.0 minutes		
	Difenoconazole – About 3.3		
Total analysis time	5 minutes + 1.0 minute as post time		
Mass scan parameters			
Ion mode	ESI, positive polarity		
Scan type	MRM		
Dry gas temperature (°C)	300		
Dry gas flow (L/min)	8		
Nebuliser (psi)	40		
Sheath Gas Temp (°C)	400		
Sheath Gas Flow (L/min)	12		
Capillary current (V)	3000		
Nozzle Voltage/Charging (V)	1500		
Compound	Prothioconazole	Prothioconazole-desthio	Difenoconazole
Precursor ion (m/z) - (fragmentor, V)	342 – (115)	312.2 – (100)	406.2 – (115)
Product ions (m/z) – (Collision Energy, V)	quantifier 100.0 – (25)	quantifier 125.0 – (38)	quantifier 251.1 – (22)
	qualifier 264.0 – (21)	qualifier 69.8 (*) - (26)	qualifier 188.4 - (54)
Dwell time (msec)	50		

(*) Note: for the analyte Prothioconazole-desthio it was not possible to find both the product ions with m/z > 100, as required by SANTE/2020/12830, rev.2; this analyte fragmented only in two product ions, one of them with m/z < 100.

Results and discussions

Calibration (matrix-matched standard solutions)

Prothioconazole, Prothioconazole-desthio and difenoconazole linearity was checked by a 5-points calibration curve using the standard solutions. The analytical calibration was prepared in the range 0.500 – 50.0 µg/L, as nominal concentrations, for each analyte (corresponding to 0.0020 – 0.200 mg/kg). Linearity was considered acceptable since the regression residuals plots were randomly distributed.

Recovery (Accuracy) and Repeatability (Precision)

Analyte	Prothioconazole		Prothioconazole-desthio		Difenoconazole	
Ion	Primary (342 m/z -> 100 m/z)	Confirmatory (342 m/z -> 264 m/z)	Primary (312 m/z -> 125 m/z)	Confirmatory (312 m/z -> 69.8 m/z)	Primary (406.2 m/z -> 251.1 m/z)	Confirmatory (406.2 m/z -> 188.4 m/z)
Linear calibration	Y=91.5x+123.3 r ² =0.99708	y=47.2+48.4 r ² =0.99879	Y=189.3x+190.6 r ² =0.99991	y=625.8+753.8 r ² =0.99997	y=676.5+4755.5 r ² =0.99979	y=71.4 + 517.8 r ² =0.99976

% Re-cov-ery ± (% RSD)	LOQ = 76.4 ± 3.2 % 10xLOQ= 98.1 ± 9.3 %	LOQ = 86.3 ± 2.7 % 10xLOQ= 96.5 ± 9.7 %	LOQ = 107.4 ± 10.0 % 10xLOQ= 89.1 ± 14.7 %	LOQ = 105.1 ± 16.5 % 10xLOQ= 87.2 ± 15.0 %	LOQ = 94.5 ± 5.6 % 10xLOQ= 92.4 ± 3.8 %	LOQ = 92.9 ± 10.5 % 10xLOQ= 89.9 ± 4.7 %
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Matrix effect

The matrix effect measured resulted not significant (lower than 20%) in Primary GLP study code LBN-0092-2023. In any case, matrix matched standard solutions were used to compensate the slight matrix effect.

Matrix Effects Results		
GLP Primary study	Analyte	Matrix Effect (%)
Primary GLP study code LBN-0092-2023	Prothioconazole	- 0.06 % (not significant)
	Prothioconazole-desthio	-10.5 % (not significant)
	Difenoconazole	- 7.93 % (not significant)

Limit of detection (LOD)

The limit of detection (LOD) is the lowest calibration level standard solution at 0.500 µg/L (corresponding to 0.002 mg/kg) of each analyte ($\leq 30\%$ LOQ).

Limit of quantification (LOQ)

The limit of quantification (LOQ) is the lowest fortification level solution at 0.01 mg/kg of each analyte in the honey samples.

Confirmation:

The Prothioconazole, Prothioconazole-desthio and difenoconazole determination was conducted by HPLC-MS/MS in MRM mode, monitoring two MS/MS ion mass transitions: 342.0 → 100.0, 312.2 → 125.0 and 406.2 → 251.1 primary detection for quantification, 342.0 → 264.0, 312.2 → 69.8(*) and 406.2 → 188.4 for qualitative purpose (confirmation).

(*) Note: for the analyte Prothioconazole-desthio it was not possible to find both the product ions with $m/z > 100$, as required by SANTE/2020/12830, rev.2; this analyte fragmented only in two product ions, one of them with $m/z < 100$.

Stability:

Samples extract: Verified for all the analytes for 5 days at $5 \pm 3^\circ\text{C}$ in the dark (stable with recovery values ranging from 60 to 120%).

Standard solutions: The stability of the Prothioconazole-desthio and Difenoconazole in the stock standard solutions was verified for 96 days at $5 \pm 3^\circ\text{C}$ in the dark for stock solutions in acetonitrile (stable with difference between stored and fresh solution lower than 10%).

Description of the method for the quantification of triazole-derivative metabolites (TDMs)

Recovery and Repeatability (Precision)

The recovery and the repeatability (as precision, % RSD) of the analytical method were determined using freshly fortified control samples of honey. Since the validation of the linearity and calibration test was not a requirement, but the calibration is necessary to quantify the fortified matrix samples, it is presented below how the calibration was prepared and the obtained results. Fortified samples were quantified using the equation of the calibration curve, with the standard solutions injected in the same run with the

samples: five working standard solutions (from WSS1 to WSS5) were prepared in solvent and they were injected before the fortification levels. Fortification levels were chosen at LOQ 0.01 mg/kg and at 10xLOQ 0.1 mg/kg in honey, as nominal concentrations.

Method for the quantification of triazole-derivative metabolites (TDMs)									
Column	HPLC column, Hypercarb, 5 μm, 100 x 2.1 mm i.d.; Internal code LCN 421 (or equivalent)								
Detector	MS Triple quadrupole (Scan in MRM mode)								
Eluent A	Water with acetic acid 1%								
Eluent B	Methanol with acetic acid 1%								
Eluent flow	0.6 mL/min								
Elution mode	gradient condition								
Solvent composition	Time (min)	% A			% B				
	0	100			0				
	1	100			0				
	6	10			90				
	7	10			90				
	7.1	100			0				
Column temperature	40°C								
Volume of injection	2 μL								
Retention time	TRZ - About 0.90 minutes								
	TAL - About 1.05 minutes								
	TLA - About 3.50 minutes								
	TAA - About 3.80 minutes								
Total analysis time	10 minutes								
Mass scan parameters									
Ion mode	ESI, positive polarity								
Scan type	MRM								
Curtain gas flow (mL/min)	30.00								
Gas 1 flow (mL/min)	55.00								
Gas 2 flow (mL/min)	65.00								
Gas Temperature (°C)	500.00								
Capillary current (V)	3500.00								
MRM transitions parameters									
Compound	Precursor ion (m/z)	Product ion (m/z)	DP	EP	CE	CXP	COV	DMO	SV
TRZ	70.1	43.1 (quantifier)	90	6	28	20	-13.3	-12	3000
		70.0 (qualifier)			13	10			
TAL	157.0	70.0 (quantifier)	13	7	18	10	-0.6	-30	2500
		88.0 (qualifier)			18	10			
TLA	158.0	70.0 (quantifier)	40	10	25	10	0.15	-35	3500
		43.0 (qualifier)			55	10			
TAA	128.0	70.0 (quantifier)	60	10	24	8	-3.8	-30	3000
		73.0 (qualifier)			20	9			

Compound	Precursor ion (m/z)	Product ion (m/z)	DP	EP	CE	CXP	COV	DMO	SV
TRZ ISTD	75.0	46.0	90	6	28	20	-13.1	-12	3000
TAL ISTD	159.0	72.0	20	10	20	10	1.15	-35	3400
TLA ISTD	163.0	75.0	40	10	25	10	0.15	-35	3500
TAA ISTD	133.0	75.0	60	10	26	8	-3.6	-30	3000
TAL ISTD_2 (*)	159.0	89.0	20	10	18	10	1.15	-35	3400

TRZ: 1,2,4-Triazole; TAL: 1,2,4-Triazole Alanine; TLA: 1,2,4-Triazole lactic acid; TAA: 1,2,4-Triazole

acetic acid; DP: declustering potential. EP: entry potential. CE: collision energy. CXP: collision cell exit potential. COV: compensation voltage. DMO: DMS offset. SV: separation voltage.
(*) Note: this transition will not be taken into account for calculations. It is used only as confirmatory for TAL ISTD.

Results and discussions

Confirmation:

The analytical method for the TDMs (triazole-derivative metabolites) determination consisted in the addition of a proper amount of ILIS (isotope-labelled internal standards) and the analysis of the samples extract with HPLC-MS/MS, equipped with a differential mobility separation device (DMS), in MRM mode, monitoring the MS/MS ion mass transitions:

- 1,2,4-Triazole: 70.1 → 43.1 (quantification) 70.1 → 70 (confirmation)

- Triazole Alanine: 157 → 70 (quantification) 157 → 88 (confirmation)

- Triazole Lactic Acid: 158 → 70 (quantification) 158 → 43 (confirmation)

- Triazole Acetic Acid: 128 → 70 (quantification) 128 → 73 (confirmation)

Calibration (working standard solutions)

The triazole-derivative metabolites linearity was checked by a 5-points calibration curve using the standard solutions. The analytical calibration was prepared in the range 0.500 – 50.0 µg/L, as nominal concentrations, for each analyte (corresponding to 0.0020 – 0.200 mg/kg). Linearity was considered acceptable since the regression residuals plots were randomly distributed

Recovery (Accuracy) and Repeatability (Precision)

Analyte	1,2,4-Triazole		1,2,4-Triazole acetic acid		1,2,4-Triazole Alanine		1,2,4-Triazole lactic acid	
Ion	Primary (primary, 70.1 m/z -> 43.1 m/z)	Confirmatory (70.1 m/z -> 70.0 m/z)	Primary (primary, 128.0 m/z -> 70.0 m/z)	Confirmatory (128.0 m/z -> 73.0 m/z)	Primary (157.0 m/z -> 70.0 m/z)	Confirmatory (157.0 m/z -> 88.0 m/z)	Primary (primary, 158.0 m/z -> 70.0 m/z)	Confirmatory (158.0 m/z -> 43.0 m/z)
Linear calibration	y=0.0622 - 0.0077x r ² =0.99994	y=0.5586+0.0687x r ² =0.99999	y=0.1130 - 0.0223x r ² =0.99989	y=0.0049 - 0.0216x r ² =0.99968	y=1.3679x+77.00 r ² =0.99816	y=0.7341+0.3037x r ² =0.99926	Y=0.0171x+0.0009 r ² =0.99991	y=0.0021+0.0011x r ² =0.99869
% Recovery ± RSD	LOQ = 109.9 ± 6.1 % 10xLOQ = 101.4 ± 2.8 %	LOQ = 101.8 ± 3.3 % 10xLOQ = 101.5 ± 2.0 %	LOQ = 104.2 ± 4.4 % 10xLOQ = 97.6 ± 1.4 %	LOQ = 98.1 ± 7.1 % 10xLOQ = 94.6 ± 4.8 %	LOQ = 80.5 ± 9.1 % 10xLOQ = 85.8 ± 4.7 %	LOQ = 80.5 ± 9.1 % 10xLOQ = 87.1 ± 3.9 %	LOQ = 103 ± 5.8 % 10xLOQ = 102.9 ± 2.5 %	LOQ = 111.9 ± 5.8 % 10xLOQ = 112.1 ± 2.6 %

Matrix effect

The matrix effect measured resulted not significant (lower than 20%) in Primary GLP study code LBN-0092-2023. In any case, matrix matched standard solutions were used to compensate the slight matrix effect.

Matrix Effects Results		
GLP Primary study	Analyte	Matrix Effect (%)
Primary GLP study code LBN-0093-2023	1,2,4-Triazole	+ 2.4 % (not significant)
	Triazole Alanine	-7.2 % (not significant)
	Triazole Lactic Acid	+ 2.1 % (not significant)
	Triazole Acetic Acid	- 2.5 % (not significant)

Limit of Detection

The limit of detection (LOD) is the lowest calibration level standard solution at 0.500 µg/L (corresponding to 0.002 mg/kg) of each analyte ($\leq 30\%$ LOQ).

Limit of Quantification

The limit of quantification (LOQ) is the lowest fortification level solution at 0.01 mg/kg of each analyte in the honey samples.

Stability:

Analytes in the samples extract: Not required since Isotope-Labelled Internal Standards (ILIS) were used.

Stability of the analytes in the standard solutions: Verified for 78 days at $5 \pm 3^\circ\text{C}$ in the dark for stock solutions in water (stable with difference between stored and fresh solution lower than 10%).

Conclusion

The primary method, validated in GLP studies Code LBN-0092-2023 (Determination of Difenoconazole, Prothioconazole and Prothioconazole-desthio in Honey) and LBN-0093-2023 (Determination of Triazole Derivative Metabolites (TDMs) in Honey) performed by LabAnalysis s.r.l., was fully validated. The study CH - 0859-2023 validated and confirmed the LOQ of the primary method according to the guideline SANTE/2020/12830 rev. 2 dated 14/02/23 and it can be considered suitable for the determination of Prothioconazole Prothioconazole-desthio, Difenoconazole 1,2,4-triazole, 1,2,4-triazole alanine, 1,2,4-triazole lactic acid and 1,2,4-triazole acetic acid residues in the tested matrix (honey).

A 2.1.1.3 Description of Methods for the Analysis of Soil (KCP 5.2)

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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The aim of this study is to develop and validate an analytical method for the determination of difenoconazole residues in soil samples of Difenoconazole 250 g/L EC greener – IN005B1570 that will come from the ecotoxicological tests.

Reference: KCP 5.1.2/011

Report	Validation of the Analytical Method for the Determination of Difenoconazole residues in soil samples of Difenoconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests Garagna, D. 2021 ChemService Study No. 0368/2021
Guideline(s):	Yes <ul style="list-style-type: none">- SANTE/2020/12830, rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.- Soil samples will be prepared according to the following guidelines.- OECD Guideline for Testing of Chemicals No. 222. "Earthworm Reproduction Test (Eisenia fetida/ Eisenia andrei)", 2016.- OECD Guideline for Testing of Chemicals No. 226. "Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil", 2016.- OECD Guideline for Testing of Chemicals, No. 232, "Collembolan reproduction test in soil", 2016.- Method validation will be performed as described in the "Standard Operating Procedures" in force at the involved laboratories.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Principle of the method

The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.

Transitions (MS/MS):

- 406.1 → 251 (quantifier);
- 406.1 → 337 and 406.1 → 188 (qualifiers)

Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area.

Reference Material

- Difenoconazole analytical standard

Instrumental settings:

Chromatographic conditions	
Column	HPLC column, Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d.; Internal code LCN 382 (or equivalent)
Detector	MS Triple quadrupole (Scan in MRM mode)
Column temperature	Not Controlled
Eluent A	Water / formic acid 0.1% / ammonium formate 10

	mM		
Eluent B	Acetonitrile		
Eluent flow	0.7 mL/min		
Elution mode	gradient condition		
Mixture	% A	% B	Time (min)
	30	70	0
	15	85	10
Volume of injection	5 µL		
Retention time	Approximately 4.1 minutes		
Total analysis time	10 minutes		
<u>Mass scan parameters</u>			
Compound	Difenoconazole		
Ion mode	ESI, positive polarity		
Scan type	MRM		
Electro multiplier voltage (V)	300		
Dry gas temperature (°C)	300		
Dry gas flow (L/min)	10		
Nebuliser (psi)	40		
Capillary current (V)	4000		
Precursor ion (m/z) - (fragmentor, V)	406.1 - (90)		
Product ions (m/z) - (Collision Energy, V)	quantifier 251 – (25) qualifiers 337 – (15), 188 (40)		
Dwell time (msec)	200		

Results and discussions

Summary of obtained results:

Parameter	Acceptability criteria
Matrix Effect	< ±20%
result	+4.2 %
Selectivity / Specificity	untreated blank < 30% LOQ
result	+1 %
Linearity / Calibration	$r \geq 0.99$
result	Quantifier transition m/z 406.1 → m/z 251
	Range 1.9 – 194.8 µg/L, $r = 0.99986$
	Corresponding to range 3.9 – 389.6 µg/kg, $r = 0.99986$
	Qualifier 1 transition m/z 406.1 → m/z 337
	Range 1.9 – 194.8 µg/L, $r = 0.99994$
	Corresponding to range 3.9 – 389.6 µg/kg, $r = 0.99994$
	Qualifier 2 transition m/z 406.1 → m/z 188
	Range 1.9 – 194.8 µg/L, $r = 0.99990$

	Corresponding to range 3.9 – 389.6 µg/kg, r = 0.99990
LOD	Lowest calibration level
result	1.9 µg/L
LOQ	Lowest fortified level
result	42.3 µg/kg
Stability of final extract	Recovery Mean between 70% – 120%
Result (3 days)	Low level: 72.2%
Stability of standard	< ±10%
Result (3 days)	7.3%

Repeatability (Precision) Recovery (Accuracy)		
Matrix	Demineralized water	
Fortification level	Low (mg/L)	High (mg/L)
Nominal	45.4	227.00
Corrected*	42.3	215.25
Mean found	30.4	181.18
Recovery	Mean between 70% – 120%	
result	72.0	84.2
Repeatability	as precision RSD % ≤ 20%	
result	1.2	3.3

(*) corrected for exact test item weighed amount during fortification solution preparation.

Qualifier Transitions

Qualifier 1 Recovery and Precision

Sample ID	Area	Found ug/L	Found ug/kg	Added ug/kg	Recovery %
Blank-Matrix_A1	49	n.d.	n.d.	0.0	-
Blank-Matrix_A2	57	n.d.	n.d.	0.0	-
SpikeLow_A	2148	16.3	32.1	43.4	73.9
SpikeLow_B	2140	16.2	31.3	42.5	73.7
SpikeLow_C	2135	16.2	30.2	41.1	73.5
SpikeLow_D	2184	16.6	32.1	42.7	75.2
SpikeLow_E	2109	16.0	30.3	41.8	72.6
Mean Value:			31.2	42.3	73.8
Standard Deviation S.D.			0.9	0.9	0.9
Relative Standard Deviation RSD%			2.9%	2.1%	1.3%

A recovery of 73.8 and a precision of 1.3% was determined for qualifier 1.

Qualifier 2 recovery and precision

Sample ID	Area	Found ug/L	Found ug/kg	Added ug/kg	Recovery %
Blank-Matrix_A1	31	n.d.	n.d.	0.0	-
Blank-Matrix_A2	9	n.d.	n.d.	0.0	-

SpikeLow_A	1340	15.4	30.3	43.4	69.8
SpikeLow_B	1372	15.7	30.4	42.5	71.5
SpikeLow_C	1353	15.5	28.9	41.1	70.5
SpikeLow_D	1402	16.1	31.2	42.7	73.1
SpikeLow_E	1372	15.7	29.9	41.8	71.5
Mean Value:			30.1	42.3	71.3
Standard Deviation S.D.			0.8	0.9	1.2
Relative Standard Deviation RSD%			2.7%	2.1%	1.7%

A recovery of 71.3 and a precision of 1.7% was determined for qualifier 2.

Conclusion

A mean recovery of 70 % - 120 % with a Relative Standard Deviation lower than 20 % was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.4 Description of Methods for the Analysis of Water (KCP 5.2)

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2
	This method was used for pre-registration purposes and is suitable for these purposes.

The aim of this study is to develop and validate an analytical method for the determination of dif-enoconazole content in stock solutions prepared with Difenoconazole 250 g/L EC greener – IN005B1570 that will come from the ecotoxicological tests.

Reference: KCP 5.1.2./12

Report Validation of the Analytical Method for the Determination of Difenoconazole content in Stock Solutions of Difenoconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests
Garagna, D. 2021
ChemService Study No. 0782/2021

Guideline(s): Yes

- SANTE/2020/12830, rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.
- Stock solutions were prepared according to the following guideline.
- OECD Guideline for Testing of Chemicals No. 208, “Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test”, 2006.
- OECD Guideline for Testing of Chemicals No. 227, “Terrestrial Plant Test: Vegetative Vigour Test”, 2006.
- Method validation was performed as described in the “Standard Operating Procedures” in force at the involved laboratories.

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Principle of the method

The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.

Transitions (MS/MS):

- Difenoconazole
- 406.1 → 250.9 (quantifier);
- 406.1 → 336.9 and 406.1 → 188 (qualifiers)

Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area.

Reference Material

A - Difenoconazole

B – test item.

Instrumental settings:

<u>Chromatographic conditions</u>			
Column	HPLC column, Zorbax Eclipse Plus C18, 3.5 μm, 100 x 4.6 mm i.d.; Internal code LCN 307 (or equivalent)		
Detector	MS Triple quadrupole (Scan in MRM mode)		
Column temperature	Not Controlled		
Eluent A	Water / formic acid 0.1% / ammonium formate 10 mM		
Eluent B	Acetonitrile		
Eluent flow	0.7 mL/min		
Elution mode	gradient condition		
Mixture	% A	% B	Time (min)
	40	60	0.11
	40	60	8.00
	10	90	8.10
	10	90	12.00
	40	60	16.00
	40	60	18.00
Volume of injection	5 μL		
Retention time	Approximately 7.4 minutes		
Total analysis time	18 minutes		
<u>Mass scan parameters</u>			
Ion mode	ESI, positive polarity		
Scan type	MRM		

Interface temperature (°C)	300
Dry gas flow (L/min)	10
Nebulizing Gas Flow (L/min)	2.9
Compound	Difenoconazole
Precursor ion (m/z)	406.1
Product ions (m/z)	Quantifier 250.9 Collision Energy: - 25 Q1 Pre Bias - 12.0 Q3 Pre Bias - 17.0
Product ions (m/z)	Qualifier 1: 336.9 Collision Energy: - 18 Q1 Pre Bias - 12.0 Q3 Pre Bias - 23
Product ions (m/z)	Qualifier 2: 188.0 Collision Energy: - 45 Q1 Pre Bias - 12.0 Q3 Pre Bias - 19.0
Dwell time (msec)	200

Results and discussions

Summary of results:

Parameter	Acceptability criteria
Matrix Effect	< ±20%
result	-12.0 %
Selectivity / Specificity	untreated blank < 30% LOQ
result	0 %
Linearity / Calibration	$r \geq 0.99$
result	Quantifier transition m/z 406.1 → m/z 251
	Range 0.02 – 1.87 mg/L $r = 0.99335$
	Qualifier 1 transition m/z 406.1 → m/z 337
	Range 0.02 – 1.87 mg/L $r = 0.99309$
	Qualifier 2 transition m/z 406.1 → m/z 188
	Range 0.02 – 1.87 mg/L $r = 0.99325$
LOD	Lowest calibration level
result	0.02 mg/L
LOQ	Lowest fortified level
result	0.23 mg/L
Stability of final extract	Recovery Mean between 70% – 120%
result	Samples analyzed within 24h from preparation, stability not performed
Stability of standard	< ±10%
result	Standard prepared freshly and analyzed within 24h from preparation, stability not performed

Repeatability (Precision) Recovery (Accuracy)	
<u>Matrix</u>	Demineralized water

Fortification level	Low (mg/L)	High (mg/L)
Nominal	0.23	9080
Corrected*	0.23	9080.05
Mean found	0.24	10210.56
Recovery	Mean between 70% – 120%	
result	104.6	112.5
Repeatability	as precision RSD % \leq 20%	
result	7	3

(*) corrected for exact test item weighed amount during fortification solution preparation.

Conclusion

A mean recovery of 70 % - 120 % with a Relative Standard Deviation lower than 20 % was adopted as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.5 Description of Methods for the Analysis of Water (KCP 5.2)

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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The aim of this study is to develop and validate an analytical method for the determination of difenoconazole residues in aqueous samples of Difenoconazole 250 g/L EC greener – IN005B1570 that will come from the ecotoxicological tests.

Reference:	KCP 5.1.2/13
Report	Validation of the Analytical Method for the Determination of Difenoconazole residues in aqueous samples of Difenoconazole 250 g/L EC greener – IN005B1570 coming from the Ecotoxicological tests Garagna, D. 2021 ChemService Study No. 0367/2021
Guideline(s):	Yes <ul style="list-style-type: none"> - SANTE/2020/12830, rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. - Aqueous samples will be prepared according to the following guidelines. - OECD Guideline for Testing of Chemicals No. 201, “Freshwater algae and cyanobacteria growth inhibition test”, 2011. - OECD Guideline for Testing of Chemicals No. 202, “Daphnia sp., Acute Immobilization Test”, 2004.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Principle of the method

The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.

Transitions (MS/MS):

- 406.1 → 251 (quantifier);
- 406.1 → 337 and 406.1 → 188 (qualifiers)

Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area.

Reference Material

- Difenoconazole

Instrumental settings

Chromatographic conditions			
Column	HPLC column, Zorbax Eclipse Plus C18, 3.5 μm, 100 x 4.6 mm i.d.; Internal code LCN 382 (or equivalent)		
Detector	MS Triple quadrupole (Scan in MRM mode)		
Column temperature	Not Controlled		
Eluent A	Water / formic acid 0.1% / ammonium formate 10 mM		
Eluent B	Acetonitrile		
Eluent flow	0.7 mL/min		
Elution mode	gradient condition		
Mixture	% A	% B	Time (min)
	30	70	0
	15	85	10
Volume of injection	1 μL		
Retention time	Approximately 4.1 minutes		
Total analysis time	10 minutes		
Mass scan parameters			
Compound	Difenoconazole		
Ion mode	ESI, positive polarity		
Scan type	MRM		
Electro multiplier voltage (V)	300		
Dry gas temperature (°C)	300		
Dry gas flow (L/min)	7		
Nebuliser (psi)	40		
Capillary current (V)	4000		
Precursor ion (m/z) - (fragmentor, V)	406.1 - (90)		

Product ions (m/z) - (Collision Energy, V)	quantifier 251 – (25) qualifiers 337 – (15), 188 (40)
Dwell time (msec)	200

Results and discussions

Summary of results:

Parameter	Acceptability criteria	
Matrix Effect	< ±20%	
result	Reconstituted water	Algal growth medium
	-30.9%	-38.3%
Selectivity / Specificity	untreated blank < 30% LOQ	
result	Reconstituted water	Algal growth medium
	+22%	+18%
Linearity / Calibration	r ≥ 0.99	
result	Reconstituted water	Algal growth medium
	Quantifier transition m/z 406.1 → m/z 251	
	Range 1.9 – 189.1 µg/L r = 0.99928	Range 1.9 – 189.1 µg/L r = 0.99751
	Qualifier 1 transition m/z 406.1 → m/z 337	
	Range 1.9 – 189.1 µg/L r = 0.99944	Range 1.9 – 189.1 µg/L r = 0.99811
	Qualifier 2 transition m/z 406.1 → m/z 188	
	Range 1.9 – 189.1 µg/L r = 0.99912	Range 1.9 – 189.1 µg/L r = 0.99808
LOD	Lowest calibration level	
result	1.9 µg/L	
LOQ	Lowest fortified level	
result	Reconstituted water	Algal growth medium
	9.5 µg/L	9.6 µg/L
Stability of final extract	Recovery Mean between 70% – 120%	
result	Reconstituted water	Algal growth medium
	Low level: 100.9%	108.7%
Stability of standard	< ±10%	
result	Reconstituted water	Algal growth medium
	-0.6%	-2.1%

Repeatability (Precision) Recovery (Accuracy)				
Matrix	Reconstituted water		Algal growth medium	
Fortification level	Low (µg/L)	High (µg/L)	Low (µg/L)	High (µg/L)
Nominal	<u>9.1</u>	<u>2.27</u>	<u>9.1</u>	<u>6.81</u>
Corrected*	9.5	2.38	9.6	7.22
Mean found	10.8	2.51	10.8	8.46
Recovery	Mean between 70% – 120%			
result	112.8	105.4	111.8	117.2
Repeatability	as precision RSD % ≤ 20%			
result	9	4	1	4

(*) corrected for exact test item weighed amount during fortification solution preparation.

Conclusion

A mean recovery of 70 % - 120 % with a Relative Standard Deviation lower than 20 % was adopt-ed as acceptability criteria.

The results obtained concerning matrix effects, linearity, selectivity, accuracy (recovery), precision (repeatability), specificity, limit of quantification and limit of detection are in compliance with requirements reported in guideline SANTE/2020/12830 rev. 1 for the analyte.

A 2.1.1.6 Description of Methods for the Analysis of Water (KCP 5.2)

Comments of zRMS: Method is accepted

The following analytical method was used in KCP 10.3.1.2 and KCP 10.3.1.3.

Reference: KCP 5.1.2/16

Report Difenoconazole 250 g/L EC greener-IN005B1570: Validation of the Analytical Method for the Determination of Difenoconazole content in Feeding Solutions and in Aqueous Stock Solutions for Honey Bees tests Tediosi, E 2023 Study No. CH – 0102-2023

Guideline(s): Yes.

- European Commission, Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830, Rev.2 (14/02/2023).
- OECD, Guideline for the Test of chemicals No.239: “Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure”, adopted on July 2021.
- OECD, Guideline for the Test of chemicals No.245: “Honey Bee (*Apis mellifera* L.), Chronic Oral Toxicity Test (10-feeding), adopted on October 2017

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.

Transitions (MS/MS):

- 406.1 → 251.0 (quantifier)
- 406.1 → 337.0 (qualifier)

Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentration and the corresponding peak area.

Description of the method

Reference Material

- Difenoconazole analytical standard

Instrumental settings:

Chromatographic conditions			
Column	HPLC column, Luna Omega 5u PS C18 100A, 5 µm, 150 x 4.6 mm i.d.; Internal code LCN 438 (or equivalent)		
Detector	MS Triple quadrupole (Scan in MRM mode)		
Column temperature	25°C		
Eluent A	Water with formic acid 0.1% v/v		
Eluent B	Acetonitrile		
Eluent flow	0.7 mL/min		
Elution mode	gradient condition		
Mixture	% A	% B	Time (min)
	30	70	0
	5	95	10
Volume of injection	1 µL		
Retention time	Approximately 5.3 minutes		
Total analysis time	10 minutes + 3 minutes as post time		
Mass scan parameters			
Compound	Difenoconazole		
Ion mode	ESI, positive polarity		
Scan type	MRM		
Electro multiplier voltage (V)	0		
Dry gas temperature (°C)	300		
Dry gas flow (L/min)	13		
Nebuliser (psi)	45		
Sheath Gas Temp (°C)	350		
Sheath Gas Flow (L/min)	12		
Capillary current (V)	3500		
Nozzle Voltage/Charging (V)	1000		
Precursor ion (m/z) - (fragmentor, V)	406.1 - (90)		
Product ions (m/z) - (Collision Energy, V)	quantifier 251 – (25) qualifiers 337 – (15), 188 (40)		
Dwell time (msec)	200		
Cell Accelerator (V)	5		

Selectivity and Specificity

The Difenoconazole determination was conducted by HPLC-MS/MS in MRM mode, monitoring two MS/MS ion mass transitions: 406.1 → 251.0 for quantification and 406.1 → 337.0 for qualitative purpose

(confirmation).

The SANTE/2020/12830, Rev.1 guideline requires that the specificity of instrumental response to the substance presence is demonstrated.

The Blank value (untreated sample) should not exceed 30% of the LOQ.

The specificity test was conducted injecting the following samples and comparing the signals to check for the possible interference by other analytes or by the matrix.

Recovery (Accuracy) and Repeatability (Precision)

The recovery and the repeatability (as precision, % RSD) of the analytical method were determined using freshly fortified control samples of Sucrose solution and of HPLC grade water.

Fortification levels were chosen at the LOQ and at the higher concentration tested in ecotoxicological studies.

Fortified samples were quantified using the equation of the calibration curve, with the standard solutions injected in the same run with the samples (bracketing calibration): five matrix matched standard solutions (from MSS1 to MSS5) were prepared in matrix and they were injected before and after the fortification levels (i.e., two series of injections).

Bracketing calibration allowed a more precise quantification, since intensity obtained from the analysis of fortified samples were compared to intensity of standard solutions analysed immediately before and after samples, avoiding problems due to variation of instrumental response during analysis.

Matrix Effects

The assessment of matrix effects was performed by comparing the slope of the curve obtained with three working standard solutions (WSS1 – WSS3 – WSS5), prepared in solvent (acetonitrile), to the slope obtained with three matrix-matched standard solutions (MSS1 – MSS3 – MSS5), prepared in blank matrix (Water HPLC grade and 50% Sucrose solution diluted in demineralised water by a factor of 100).

Matrix effects, expressed in % enhancement or suppression of signal, are considered significant if they exceed $\pm 20\%$.

Limit of detection (LOD) and Limit of quantification

The limit of detection (LOD) is defined as the lowest calibration level standard solution, it was prepared at 0.02 mg/L as Difenoconazole nominal concentration.

The limit of quantification (LOQ) is defined as the lowest fortification level solution, and it was set at the following nominal concentrations:

- 26 mg test item/L in Sucrose solution (corresponding to 6 mg Difenoconazole/L in the sample and to a final injected solution equal to 0.06 mg Difenoconazole/L, after dilution in demineralised water by a factor of 100);
- 0.26 mg test item/L in Water HPLC grade (corresponding to 0.06 mg Difenoconazole/L).

Linearity

The analytical calibration was performed by injecting, in duplicate determination, five matrix matched

standard solutions (MSS) prepared in matrix (Water HPLC grade and 50% Sucrose solution diluted in de-mineralised water by a factor of 100).

Linear regression analysis was performed using the least squares method; the correlation coefficient was calculated using regression analysis.

Final extract and Standard Stability

Extract stability:

The stability of the analyte in the final extracts is proven if the recoveries in the fortified samples are within the acceptable range of 70 - 120%, measured against freshly prepared standards.

Standard stability:

The stability of an existing standard (old) was checked by preparing a new standard solution (fresh) and comparing the responses. The means from 5 replicate measurements for each of the two solutions should not differ by more than 10%.

Results and discussions

Summary of obtained results:

Parameter	Acceptability criteria	
Matrix Effect	< ±20%	
Matrix	Sucrose Solution	Water HPLC Grade
result	-22.4 % (significant)	-2.0% (not significant)
	Matrix-matched standard solutions are prepared	
Selectivity / Specificity	untreated blank < 30% LOQ	
Matrix	Sucrose solution	Water HPLC Grade
result	0 % (specific)	0% (specific)
Linearity / Calibration	$r \geq 0.99$	
Matrix	Sucrose Solution	Water HPLC Grade
result	Quantifier transition m/z 406.1 → m/z 251 Range 0.02 – 181 mg/L, $r = 0.99839$ Qualifier transition m/z 406.1 → m/z 337 Range 0.02 – 181 mg/L, $r = 0.99867$	
	Range 0.02 – 181 mg/L, $r = 0.99789$	Range 0.02 – 181 mg/L, $r = 0.99816$
LOD	Lowest calibration level	
Matrix	Sucrose Solution	Water HPLC Grade
result	0.02 mg/L	0.02 mg/L
LOQ	Lowest fortified level	
Matrix	Sucrose Solution	Water HPLC Grade
result	6.04 mg/L (diluted: 0.06 mg/L)	0.06 mg/L
Stability of final extract	Analysis performed within 24 hours from preparation; stability check not performed	
Stability of standard	Standard prepared freshly; stability check not performed	

Repeatability (Precision) Recovery (Accuracy)				
Matrix	Sucrose Solution		Water HPLC Grade	
Fortification level	Low (mg/L)	High (mg/L)	Low (mg/L)	High (mg/L)
Nominal	6	2250	0.06	2250
Corrected*	6.04	2275.65	0.06	2256.75
Mean found	6.54	2184.79	0.05	1693.40
Recovery	Mean between 70% – 120%			
result	108.2	96.0	93.1	75.0
Repeatability	as precision RSD % \leq 20%			
result	3	7	15	2

(*) corrected for exact test item weighed amount during fortification solution preparation.

Conclusion

Due to the obtained results, the analytical method No. 0102-2023 can be considered fully validated according to the SANTE/2020/12830 rev. 1 dated 24/02/21 and it can be considered suitable for the determination of Difenoconazole residues in the ecotoxicological matrices investigated (Sucrose solution and HPLC grade water).

A 2.1.1.6.1.1 Method validation 3

Comments of zRMS:	The method is accepted according to SANTE/2020/12830, Rev. 2 This method was used for pre-registration purposes and is suitable for these purposes.
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The aim of this study is to develop and validate an analytical method for the determination of difenoconazole residues in aqueous samples of Difenoconazole 250 g/L EC greener – IN005B1570 that will come from the equipment utilization (cleaning procedure).

Reference: KCP 5.2

Report IN005B1570: equipment cleaning procedure
Longhi, D. 2022
Study No. GLP-STUDY-LBN-0040-2022

Guideline(s): Yes

- Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice and Compliance Monitoring (Monograph 11, The Role and Responsibilities of the Sponsor in the Application of the Principles of GLP) OECD ENV/MC/CHEM(98)16.
-
- SANTE/2020/12830 Rev.1 dated 24 February 2021: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.
- PSD Efficacy Guideline 302 “Cleaning Application Equipment – Efficacy Aspects” (September 2005)
- PSD Efficacy Guideline 305 “Cleaning Application Equipment – Small scale jar test protocol” (December 2004)
- European and Mediterranean Plant Protection Organization (2016 OEPP/EPPO, Bulletin OEPP/EPPO Bulletin 46, 371–378), PP 1/292

(1) "Cleaning pesticide application equipment (PAE) – efficacy aspects"

- GLP-STUDY-21-31, "Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-dethio in wheat, barley, oilseed rape and processed commodities", Test Facility: LabAnalysis srl, Study Director: Diego Longhi

Deviations: No
 GLP: Yes
 Acceptability: Yes

Materials and methods

The determination of Difenoconazole residues is performed by HPLC-UV.

Reference Material

- Difenoconazole analytical standard

Instrumental settings:

Chromatographic conditions			
Model	Agilent HPLC 1290 Infinity II		
Column	Agilent Poroshell 120 EC-C18, 4 μm, 4.6 x 100 mm		
Column temperature	40°C		
Flow	1 mL/min		
Injection volume	10 μL		
Mobile phase A	Deionised water		
Mobile phase B	Acetonitrile		
Elution	Gradient of the following composition:		
	Time (min)	% A	% B
	0	50	50
	0.5	50	50
	5	5	95
	8	5	95
Stop time	8 min		
Post time	2.5 min		
UV detector wavelength	218 nm		
Confirmatory (included only the difference compared to primary)			
Column for confirmatory	Waters XSelect HSS PFP, 3.5 μm, 4.6 x 150 mm		
Elution	isocratic elution (water/acetonitrile 35:65)		

Sample preparation

Tank mix preparation: 300 mL of water (CIPAC D) was placed into a beaker and stirred. Afterwards, an amount of 1.5 mL of IN005B1570 – Difenoconazole 250 g/L EC greener was added. After stirring, the solution was shared in 100 mL aliquots and poured into three polyethylene bottles (about 45 mm in diameter and 90 mm high). The bottles are topped and let at room temperature overnight.

The test item was used to prepare a solution at a concentration equal to the maximum one reported in the GAP table.

The maximum concentration includes in GAP was used.

Tank cleaning procedure: After the overnight standing of the test item, a triple rinse cleanout procedure without tank-cleaner was carried out:

- i. Each bottle was inverted twice to re-suspend any settled material. The tank mix was then discarded.
- ii. 10 mL of tap water were added. The bottle as inverted twice and the water was then discarded.
- iii. the previous step is repeated twice
- v. 10 mL of acetonitrile were added to the bottle. It was shaken to coat all surfaces. The acetonitrile was used to extract residual pesticide from the bottle surfaces.
- vi. The acetonitrile sample was analysed by HPLC.

Results and discussions

Recovery (Accuracy) and Repeatability (Precision)

Recovery and precision were verified by means of recovery tests carried out at the following spiking levels:

- 1.00 mg/L, corresponding to the target LOQ

- 10.0 mg/L, corresponding to 10xLOQ

The recovery tests were carried out adding known amounts of analyte in 100 mL PE (polyethylene) bottles. Once evaporated the solvent, the bottles were washed with 10 mL of acetonitrile each. 2 untreated tests were carried out.

The mean recoveries per level found for both primary and confirmatory determination were in compliance with the following requirements:

- Recoveries range: 70-120%

- RSD: $\leq 20\%$

Preparation of the fortified samples

Sample code	Spiking level (mg/L)	Added volume (μL)	Rinsing acetonitrile volume (mL)	Expected concentration in the final extract (mg/L)	Analysis date (dd/mm/yyyy)
22-40-01	Untreated	0	10	0	08/08/2022
22-40-02			10		
22-40-03			10		
22-40-04	1.00 (LOQ)	100 μL of so- lution A (100 mg/L)	10	1.00	
22-40-05			10		
22-40-06			10		
22-40-07			10		
22-40-08			10		
22-40-09	10.0(10xLOQ)	100 μL of dif- enoconazole stock solution (1001 mg/L)	10	10.0	
22-40-10			10		
22-40-11			10		
22-40-12			10		

Recovery and precision results

Primary determination						
Difenoconazole						
Specimen identification	Fortification level	Measured in the sample	Recovery (100%)	Mean recovery	SD	%RSD

		(mg/L)		(100%)		
22-40-01	Untreated	0.0000	--	--	--	--
22-40-02	Untreated	0.0000	--	--	--	--
22-40-03	LOQ	0.990033	99.0	101.1	1.7	1.6
22-40-04	LOQ	1.00417	100.4			
22-40-05	LOQ	1.03479	103.5			
22-40-06	LOQ	1.00808	100.8			
22-40-07	LOQ	1.01748	101.7			
22-40-08	10xLOQ	10.57500	105.6	103.5	1.2	1.2
22-40-09	10xLOQ	10.34130	103.3			
22-40-10	10xLOQ	10.31109	103.0			
22-40-11	10xLOQ	10.31554	103.1			
22-40-12	10xLOQ	10.26298	102.5			
Overall				102.3	1.9	1.8

Specificity (Selectivity)

The selectivity was evaluated comparing the following chromatograms in order to assess the presence or absence of interfering signals:

- acetonitrile coming from the rinsing of a PE bottle
- acetonitrile coming from the rinsing of a PE bottle treated with analyte at LOQ
- reference solutions at the LOQ level.

No interfering signals were detected in the untreated sample in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements.

The method was found to be selective for the determination of the analyte in the tested matrices for both the primary and the confirmatory determination.

Matrix effect

Assessment of matrix effect was performed comparing the analyte response of one individual standard at L4 (10 mg/L) prepared in solvent to one prepared in blank matrix (acetonitrile coming from the washing of a PE bottle with 10 mL) at the same concentration.

Analyte	Area L4 standard in solvent (acetonitrile)	Area L4 standard in matrix (acetonitrile after rinsing)	Ratio matrix-matched/Solvent (%)	Matrix effect (%)
Difenoconazole	313.07794	315.79471	100.9	+0.9

The matrix effect is considered not significant according to the SANTE/2020/12830 rev.1 guideline.

Limit of detection (LOD) and Limit of quantification (LOQ)

Matrix	LOD concentration	% of LOQ	Difenoconazole	
			S/N primary determination	S/N confirmatory determination
Acetonitrile	0.300 mg/L	20%	104.0	59.2

Confirmation

A confirmatory HPLC-UV determination was carried out analysing the same extracts analysed for the primary determination with different chromatographic conditions, in detail:

- a different stationary phase: a pentafluorophenyl-based stationary phase (column Waters XSelect HSS PFP, 3.5 µm, 4.6 mm X 150 mm), instead of a C-18.

- a different elution: isocratic elution (water/acetonitrile 35:65) instead of a gradient elution

The recovery and precision data for the confirmatory determination are reported here below.

Confirmatory determination						
Difenoconazole						
Specimen identification	Fortification level	Measured in the sample (mg/L)	Recovery (100%)	Mean recovery (100%)	SD	%RSD
22-40-01	Untreated	0.0000	--	--	--	--
22-40-02	Untreated	0.0000	--	--	--	--
22-40-03	LOQ	0.969734	97.0	99.2	1.5	1.5
22-40-04	LOQ	0.985521	98.6			
22-40-05	LOQ	1.005610	100.6			
22-40-06	LOQ	0.994634	99.5			
22-40-07	LOQ	1.003680	100.4			
22-40-08	10xLOQ	10.50123	104.9	102.8	1.2	1.2
22-40-09	10xLOQ	10.26026	102.5			
22-40-10	10xLOQ	10.24670	102.4			
22-40-11	10xLOQ	10.25236	102.4			
22-40-12	10xLOQ	10.20420	101.9			
Overall				101.0	2.3	2.3

Conclusion

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 1 for the determination of Difenoconazole in the equipment cleaning procedure.

Appendix 3 Additional information provided by the Applicant

Statement prepared by LabAnalysis Srl

zRMS: statement is accepted

Statement on the extraction efficiency for studies GLP-STUDY-21-31, GLP-STUDY-21-32, GLP-STUDY-21-108, GLP-STUDY-21-109

This statement was produced to provide justification about the extraction efficiency of the analytical methods validated in the following studies:

- *GLP-STUDY-21-31: Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities*
- *GLP-STUDY-21-32: Validation of an analytical method for the quantification of Difenoconazole in apple, carrot, tomato and processed commodities*
- *GLP-STUDY-21-108: Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities*
- *GLP-STUDY-21-109: Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in apple, carrot, tomato and processed commodities*

The procedures used in the studies GLP-STUDY-21-31 and GLP-STUDY-21-32 to extract Difenoconazole and Prothioconazole-desthio from the plant matrices were based on the QuEChERS method, that consist in a solid-liquid extraction using acetonitrile and in a separation from the water contained in the sample using a mixture of salts (sodium chloride, magnesium sulphate).

The extraction efficiency was not demonstrated in that studies for the following reasons:

- the extraction efficiency of QuEChERS method or acetonitrile was already demonstrated in the RAR of Prothioconazole
- acetonitrile (the extraction solvent used by QuEChERS method before the phase separation achieved with the addition of salts) was used as extraction systems in several residue and metabolism study accepted in the RAR of Prothioconazole and Difenoconazole.

The procedures used in the studies GLP-STUDY-21-108 and GLP-STUDY-21-109 to extract the triazole derivative metabolites (TDM) 1,2,4-triazole, triazole-alanine, triazole-acetic acid, triazole-lactic acid from the plant matrices were made using 1% formic acid in methanol/water, in accordance to the EURL SRM (EU Reference Laboratories for Residues of Pesticides) method QuPPE "Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC or IC MS/MS Measurement I. Food of Plant Origin (QuPPE PO Method) (Version 12)".

The extraction efficiency was not demonstrated in that studies for the following reasons:

- the method used was an official single residue monitoring method
- the use of the selected solvent (acidic methanol, in the presence of water that is contained in the matrix or was added for the dried ones) was supported by methods already accepted in the Prothioconazole RAR
- since the applicant was not able to provide metabolism study based on TDMs as precursors (they are not an active ingredient) and since TDM are common metabolites of all the triazole-based active ingredients, the laboratory investigated the RAR of another triazole fungicide, in detail the RAR of BAS 750F (Mefentrifluconazole) - Volume 3 – B.7. In a metabolism study of this compound, methanol was used as extraction solvents. Furthermore, the extraction efficiency of an acid methanol/water extraction system was verified comparing the extracted radioactivity with the metabolism study extraction method (section B.7.2.1.4. Extraction efficiency).

Here below some references are reported.

ANALYTE: PROTHIOCONAZOLE-DESTHIO

- From RAR PROTHIOCONAZOLE - Volume 3 – B.5

The extraction efficiency using the QuEChERS method was already claimed in the following study:

Report:	KCA 4.2/12; Chambers, J.; Jarrett, H.; 2014; M-498384-01-1
Title:	Modification M018 of the analytical method 01300 (based on “QuEChERS” method) for the determination of residues of prothioconazole-desthio and iprovalicarb in wheat grain, grapes, rapeseed, dry bean and cucumber
Report No.:	VC/13/017
Document No.:	M-498384-01-1
Guideline(s):	Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC, European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of Pre-Registration data Requirements for Annex II (part A, Section 4) and Annex III (part A, section 5) of directive 91/414, SANCO/3029/99 rev. 4, 11/07/00 Guidance document on residue analytical methods, SANCO/825/00/rev. 8.1, European Commission, Directorate General Health and Consumer Protection 16/11/2010, US EPA Residue Chemistry Test Guideline OPPTS 860.1340: Residue Analytical Method
GLP/GEP:	Yes

The objective of this study is to validate an established multi-residue monitoring method (QuEChERS) for the determination of residues of prothioconazole-desthio in wheat grain, grapes (whole bunches), rapeseed (seeds), dry bean (cannellini) and cucumber (whole fruits) to fulfil the requirements according to guidance document SANCO 825/00/ rev. 8.1

Principle of the method

The method for the determination of prothioconazole-desthio is based on the “QuEChERS” procedures which involves extraction of residues with acetonitrile/water (1/1 v/v) after addition of water only for matrices with low water content (water was added for wheat grain, rapeseed and dry bean, no addition of water to grape or cucumber), addition of buffer salts to facilitate phase separation, clean-up of an aliquot by solid-phase dispersion and determination by LC-MS/MS using a Luna 100 5 C18, 150 mm length, 4.6 mm diameter column. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM).

Extraction Efficiency

The extraction efficiency was demonstrated by method 01300/M018 in KCA 4.2/18; Desmaris, F.; 2015; M-536877-02-1(study Number MR-15/117), ‘Amendment no. 1 to the final report - Cross validation of extraction methods for the determination of residues of prothioconazole-desthio in plant material by HPLC-MS/MS’ The extraction efficiency of the method was evaluated using barley grain, wheat green material, wheat straw and rape seed matrices from nature of residue metabolism studies (M-041657-01-1 and M-103268-01-2). Samples containing incurred prothioconazole-desthio residues were reanalyzed with the sample analysis procedure described above. Results obtained using the analytical method were equivalent to those obtained in the metabolism study, demonstrating the suitability of this analytical method for the determination of prothioconazole in plant matrices.

- **From RAR PROTHIOCONAZOLE Volume 3 – B.7 (AS)**

Furthermore, acetonitrile or acetonitrile/water combination (please note that water is already present in plant matrices) was used as extraction solvent in several metabolism studies, in detail:

Report:	KCA 6.6.1/02; Duah, F. K.; Kraai, M. J.; 2004
Title:	The accumulation of [triazole-3,5-14C] JAU6476 in confined rotational crops
Report No.:	200623
Guidelines:	OPPTS 860.1850; DACO 7.4.3
GLP:	Yes

Report:	KCA 6.2.1 /01; Haas, M.; Bornatsch, W., 2000
Title:	Metabolism of JAU6476 in spring wheat (after foliar application)
Report No.:	MR-198/99
Guidelines:	Not specified
GLP:	Yes

Report:	KCA 6.2.1/02; Duah, F. K.; Lopez, R. T.; 2004
Title:	The metabolism of [triazole-3,5-14 C] JAU 6476 in wheat
Report No.:	200733
Guidelines:	US EPA OPPTS 860.1300; DACO 6.3
GLP:	Yes

Report:	KCA 6.2.1/03; Haas, M., 2001
Title:	Metabolism of JAU 6476 in spring wheat after seed dressing
Report No.:	MR-467/99
Guidelines:	Not specified
GLP:	Yes

Report:	KCA 6.2.1/04; Vogeler, K.; Sakamoto, H.; Brauner, A., 1993
Title:	Metabolism of SXX 0665 in summer wheat
Report No.:	PF3906
Guidelines:	Not specified
GLP:	Yes

Report:	KCA 6.2.1/05; Haas, M., 2001
Title:	Metabolism of [phenyl-UL-14C]JAU6476 in peanuts
Report No.:	MR-193/01
Guidelines:	Not specified
GLP:	Yes

Report:	KCA 6.2.1/06; Haas, M.; 2003
Title:	Metabolism of [triazole-UL-14C]JAU6476 in peanuts
Report No.:	MR-194/02
Guidelines:	US EPA OPPTS 860.1300; Canadian PMRA Ref.: DACO 6.3; EU 91/414/EEC amended by 96/68/EC
GLP:	Yes

Report:	KCA 6.2.1/07; Beedle, E. C.; Ying, S. L.; 2004; M-001059-01-1
Title:	The metabolism of [phenyl-UL-14C]JAU6476 in sugar beets
Report No.:	200466
Guidelines:	EPA Ref.: OPPTS 860.1300, Nature of the Residue - Plants
GLP:	Yes

Report:	KCA 6.2.1/08; Beedle, E. C.; Ying, S. L.; 2004
Title:	The metabolism of [triazole-UL-14C]JAU6476 in sugar beets
Report No.:	200467
Guidelines:	EPA Ref.: OPPTS 860.1300, Nature of the Residue - Plants

ANALYTE: DIFENOCONAZOLE

- From RAR DIFENOCONAZOLE Volume 3 – B.5

The extraction efficiency in acetonitrile (the same solvent used in QuEChERS method to carry out the extraction before the separation from water achieved through the addition of the salts) was already demonstrated in the following study:

Data point addressed:	CA 4.2.1 (a)/02
Author(s) (year):	Gasso-Brown D, (2015).
Title:	Difenoconazole – Independent Laboratory Validation of the QuEChERS Method for the Determination of Residues of Difenoconazole in Crop Matrices by LC-MS/MS.
Laboratory report / project Number (Doc. No.):	(Syngenta Task No. TK0208885). (Syngenta File No. CGA169374_10960).
Testing facility:	Eurofins Agrosience Services Chem Ltd, UK.
Published:	No

Principle of the Method

Sample material (tomato, oilseed rapeseed, dried broad bean and herbal infusion matrix) was extracted by shaking with acetonitrile, after the addition of a suitable volume of ultra-pure water, if necessary (taking into account the natural water content of the samples). The contents of a dispersive SPE citrate extraction tube (containing magnesium sulphate, sodium chloride, trisodium citrate dehydrate and disodium hydrogenocitrate sesquihydrate) was added to each extract and the mixture was shaken by hand and then centrifuged. For oilseed rape seed samples only, after centrifugation, an aliquot of the upper acetonitrile phase was transferred into a deep freezer ($\leq -15^{\circ}\text{C}$) for at least 1 hour to settle, then the centrifugation step was repeated. An amount of PSA and magnesium sulphate was added to an aliquot of the upper acetonitrile phase of all samples, and immediately shaken by hand. Following centrifugation, sample extracts were diluted with methanol/water (20/80, v/v) solution and analyzed for difenoconazole by high-performance liquid chromatography with mass-spectrometric detection (LC-MS/MS), monitoring for the primary transition (m/z 406.0-250.9) and the confirmatory transition (m/z 406.0-188.1).

Extraction Efficiency

^{14}C metabolism studies on tomato (N-0964-0700) and potato (N-0964-0400) used acetonitrile/ water to efficiently extract radioactive residues (approximately 80 – 100% of the extractable residue). As this is an identical extraction system to that used in the QuEChERS multi-residue procedure, these values demonstrate the extraction system is adequate for extracting residues of difenoconazole from crop commodities.

- From RAR DIFENOCONAZOLE Volume 3 – B.7 (AS)

The following metabolism study used acetonitrile as extraction solvent:

Previous evaluation:	Submitted in the original DAR. Considered Relevant.
Data point addressed:	CA 6.2.1/03
Author(s) (year):	Schweitzer MG (1990a)
Title:	Metabolism of phenyl- ^{14}C -CGA 169374 in spray-treated tomatoes. Novartis Crop Protection AG, Basel,
Laboratory report / project Number (Doc. No.):	Report No N-0964-0700.
Testing facility:	Battelle, Columbus, United States
Published:	No
Test guideline used:	US EPA Residues Chemistry, Series 171-4 (a) (1) & (2) (1982 and 1986), Washington, DC.
Deviations:	None
GLP:	Yes

ANALYTES: TDM

The extraction of triazole-derivative metabolites (TDM) was carried out using 1% formic acid in methanol/water, according to the official EURL SRM (EU Reference Laboratories for Residues of Pesticides) method QuPPE "Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC or IC MS/MS Measurement I. Food of Plant Origin (QuPPE PO Method) (Version 12)".

Furthermore, the use of the selected solvent (acidic methanol, in the presence of water that is contained in the matrix or was added for the dried ones) was supported by the following method already accepted in the Prothioconazole RAR:

- From RAR PROTHIOCONAZOLE - Volume 3 – B.5

Previous evaluation:	Submitted for the purpose of renewal. Considered relevant.
Data point addressed:	CA 4.1.2.5 (e)/04
Author(s) (year):	Gemrot F, (2011a)
Title:	Validation of Analytical Method GRM053.01A for the Determination of 1,2,4-Triazole, Triazole Alanine, Triazole Acetic Acid and Triazole Lactic Acid in Crops.
Laboratory report / project Number (Doc. No.):	Report No. S10-02599-REG. Syngenta File No. CGA131013_10015
Testing facility:	Syngenta, Jealott's Hill International Research Centre, UK.
Published:	No
Test guideline used:	SANCO/3029/99 rev.4, SANCO/825/00 rev.6, OECD Guidance Document ENV/JM/MONO (2007)17, EPA OPPTS 860.1340 (1996), EPA OPPTS 850.7100
Deviations:	None
GLP:	Yes

Principle of the method

The analytical method GRM053.01A was developed for the determination of residues of 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in/on crop commodities.

The analytes are extracted from crop samples with methanol/water (4/1 v/v) using a high speed homogenizer. A filtered aliquot of the raw extract is mixed with the stable isotope internal standards, concentrated to an aqueous residue and reconstituted in water.

- From RAR PROTHIOCONAZOLE - Volume 3 – B.7

The following metabolism study used acetonitrile/water and then methanol/water as extraction solvents. Since the TDMs are polar analyte, it is reasonable to suppose that a more polar solvent as methanol is at least equally efficient than acetonitrile (and this is suggested by the fact that the use of methanol after acetonitrile in the metabolism study below reported allowed the extraction of a residual 10% of radioactivity that acetonitrile was not able to extract).

Report:	KCA 6.2.1/08; Beedle, E. C.; Ying, S. L.; 2004
Title:	The metabolism of [triazole-UL-14C] JAU6476 in sugar beets
Report No.:	200467
Guidelines:	EPA Ref.: OPPTS 860.1300, Nature of the Residue - Plants
GLP:	Yes

Report:	KCA 6.6.1/02; Duah, F. K.; Kraai, M. J.; 2004
Title:	The accumulation of [triazole-3,5-14C] JAU6476 in confined rotational crops
Report No.:	200623
Guidelines:	OPPTS 860.1850; DACO 7.4.3
GLP:	Yes

- From RAR PROTHIOCONAZOLE - Volume 3 – B.7

Furthermore, the use of the selected solvent (acidic methanol, in the presence of water that is contained in the matrix or was added for the dried ones) was justified by the fact that the same solvent was accepted in the following studies accepted in the Prothioconazole RAR for rotational crops. In these studies, residues were extracted from plant material with methanol/water (4/1, v/v).

Report:	KCA 6.6.2/01; Freitag, Th.; Ruhl, S., 2012
Title:	Determination of the residues of prothioconazole in/on the field rotational crops carrot, lettuce, spring barley and winter barley after either a single application of JAU 6476 EC 250 on bare soil or sowing of spring wheat treated with JAU 6476 FS 100 followed by three spray applications with JAU 6476 EC 250 in the field in Germany
Report No.:	09-2500
Document No.:	M-426697-01-1
Guideline(s):	EU-Ref: Council Directive 91/414/EEC of July 15, 1991, Annex II, part A, section 6 and Annex III, part A, section 8 Residues in or on Treated Products, Food and Feed EC guidance working document 7029/VI/95 rev. 5 (1997-07-22) OECD Guideline for testing of Chemicals; Residues in rotational crops (limited field studies), No. 504, 8 Jan. 2007
GLP/GEP:	Yes

Report:	KCA 6.6.2/03; Freitag, Th.; Ruhl, S., 2012
Title:	Determination of the residues of prothioconazole in/on the field rotational crops turnip, lettuce, spring barley and winter barley after either a single application of JAU 6476 EC 250 on bare soil or sowing of winter wheat treated with JAU 6476 FS 100 followed by spray application with JAU 6476 EC 250 in the field in southern France
Report No.:	09-2502
Document No.:	M-426710-01-1
Guideline(s):	EU-Ref: Council Directive 91/414/EEC of July 15, 1991, Annex II, part A, section 6 and Annex III, part A, section 8 Residues in or on Treated Products, Food and Feed EC guidance working document 7029/VI/95 rev. 5 (1997-07-22) OECD Guideline for testing of Chemicals; Residues in rotational crops (limited field studies), No. 504, 8 Jan. 2007
GLP/GEP:	Yes

The following method uses as extraction solvent: methanol/water (4/1, v/v)

Report:	KCA 6.5.3/01; Freitag, Th.; Diehl, P., 2015
Title:	Determination of the residues of fluoxastrobin and prothioconazole in/on Barley, spring and the processed fractions (malt sprouts; brewer's malt; brewer's grain; hops draff; brewer's yeast; beer; pearl barley rub off; pearl barley) after spraying of Fluoxastrobin & Prothioconazole EC 200 in the field in Germany and France (South)
Report No.:	13-3401
Document No.:	RAHEN025
Guideline(s):	Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. EC guidance working document 7029/VI/95 rev. 5 (July 22, 1997). OECD 509 Adopted 2009-09-07, OECD guideline for the testing of chemicals, Crop Field Trial. OECD 508 adopted 3 October 2008, OECD Guideline for the testing of chemicals, magnitude of the pesticide residues in processed commodities. US EPA OCSPP Guideline No. 860.1520. US EPA OCSPP Guideline No. 860.1500, Crop Field Trial
GLP/GEP:	Yes

The following method uses as extraction solvent: aqueous methanol (MeOH/H₂O; 4:1, v:v)

Report:	KCA 6.5.3/05; Kraai, M. J., 2004
Title:	JAU6476 480 SC - Magnitude of the Residue in/on Wheat Grain, Wheat Aspirated Grain Fractions, and Wheat Processed Commodities
Report No.:	200521
Document No.:	J619WH02
Guideline(s):	EPA Ref.: OPPTS 860.1500, Crop Field Trials; OPPTS 860.1520, Processed Food/Feed; PMRA Ref.: DACO 7.4.1, Supervised Residue Trial Study; DACO 7.4.5, Processed Food/Feed
GLP/GEP:	Yes

- **From RAR BAS 750F (Mefentrifluconazole) - Volume 3 – B.7**

It is important to consider that TDM are common metabolites of all the triazole-based fungicides. In the case of the approved active substance Mefentrifluconazole, the extraction of the analytes with methanol from plant matrices was carried out in the following metabolism studies:

Report:	CA 6.2.1/1 Rabe U.,Bogen C., 2015 a Metabolism of 14C LS 5834378 in wheat 2015/1001872
Guidelines:	EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, Test No. 501: Metabolism in crops
GLP: <i>B.7.2.1.2. Soybean</i>	yes
Report:	CA 6.2.1/2 Thiaener J.,Bogen C., 2015 a Metabolism of 14C-BAS 750 F in soybean 2014/1224012
Guidelines:	EPA 860.1000, EPA 860.1300, EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, Test No. 501: Metabolism in crops
GLP:	yes
Report:	CA 6.2.1/3 Birk B.,Bogen C., 2015 a Metabolism of 14C-BAS 750 F in grape 2015/1073822
Guidelines:	EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, OECD 501 - Metabolism in crops (adopted January 8 2007)
GLP:	yes

In the following study, the extraction efficiency of the method named "BAS method 535/1" was verified analysing the radioactivity of sample with incurred residue coming from metabolism studies. The BAS method 535/1 use as extraction solvent acidic methanol and water, in detail methanol / water / 2 N HCl (70/25/5, v/v/v) that is not different as pH and composition from the extraction mixture described in the QuPPE method (1% formic acid in methanol/water)

B.7.2.1.4. Extraction Efficiency

Report:	CA 6.2.1/4 Birk B. et al., 2015 b Investigation of the extractability of BAS 750 F in samples from 14C plant metabolism studies 2014/1261057
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17, OECD 501
GLP:	yes