REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: GLOB1911F

Product name(s): **CURRANDO / SUBIGON / COLLECTOR**

Chemical active substance:

Difenoconazole, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: Globachem NV

Submission date: December 2020

MS Finalisation date: May 2021

Revision date: October 2021

Version history

When	What
December 2020	initial zRMS version
May 2021	Version evaluated by zRMS
October 2021	The update after comments

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5 Analytical methods

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are ~~not~~ available for the active substance(s) and relevant impurities in the plant protection product.

~~Noticed data gaps are:~~

- ~~data gap 1~~
- ~~data gap 2~~
- ~~data gap 3~~

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.
In the context of the residue analytical methods this document was not rewritten. All zRMS comments or/and corrections with regard to the residue analytical methods are within grey boxes/background.
The data are sufficient for evaluation and enforcement of all relevant MRLs/residue levels.
No data gaps were identified in the context of this authorisation request.

Commodity/crop	Supported/ Not supported
Potatoes	Supported
Sugar / fodder beets	Supported
Oilseed rape	Supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

An analytical method has been developed for the determination of the active substance Difenoconazole in GLOB1911F.

The following analytical method for the determination of the active substance in the plant protection product GLOB1911F has not previously been reviewed according to the Uniform Principles and is provided in support of this assessment.

Comments of zRMS:	The method is accepted. It meets SANCO/3030/99 rev.5 requirements and may be used for analysing active substance in the PPP.

Reference:	KCP 5.1.1
Report	Validation of the methods of determination of Difenoconazole and a specified impurity in a suspension concentrate formulation GLOB1911F containing 500 g/L Difenoconazole, in compliance with good laboratory practice, Sowle J., DNA5105, 2020a
Guideline(s):	Sanco/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The assay of Difenoconazole was performed using approximately 0.1g of Formulation. The mass of the formulation was accurately recorded, transferred to a 100 mL volumetric flask and partially made to volume with methanol. The sample was sonicated for 5 minutes, allowed to cool to room temperature, then made up to volume with methanol. The samples were then assayed by injecting each solution once into the HPLC-DAD under the following conditions:

HPLC-DAD

Instrument:	Agilent 1100 Series HPLC-DAD
Mode:	Isocratic Reverse Phase
Column:	Grace Alltima C8, 250 mm x 4.6 mm
Packing:	C8, 5 µm
Eluent:	73% Methanol, 27% Deionised Water adjusted to pH3 with Phosphoric Acid
Wavelength:	225 nm
Flow Rate:	1.0 mL/min
Injection Volume:	5 µL

Column temperature:	25°C
Data collection	Chemstation
Retention Times:	Difenoconazole Trans-Isomer approximately 10.6 minutes Difenoconazole Cis-Isomer approximately 11.3 to 11.4 minutes

LC-QToF conditions – MS Spectral analysis of active ingredient:

LC Conditions:

Instrument:	Agilent 1260 Series HPLC-DAD
Mode:	Isocratic Reverse Phase
Column:	Grace Alltima C8, 250 mm x 4.6 mm
Packing:	C8, 5 µm
Eluent:	73% Methanol, 27% Water adjusted to pH3 with Formic Acid
Wavelength:	225 nm
Flow Rate:	1.0 mL/min
Injection Volume:	5 µL
Column temperature:	25°C

MS Conditions:

Instrument:	Agilent 6500 Series Q-ToF Mass Spectrometer
Mode:	Jetstream Electrospray (ESI)
Ionisation:	Positive
MS scan range:	50-1000 m/z
MS/MS scan range:	50-500 m/z
Acquisition rate:	1 Spectra/Second
Acquisition time:	1000ms/Spectra
Retention Times:	Difenoconazole Trans-Isomer approximately 10.9 minutes Difenoconazole Cis-Isomer approximately 11.7 minutes
Gas temperature:	250°C
Drying gas flow:	7 L/min
Nebulizer:	40 psi
Shealth Gas:	250°C
Shealth Gas Flow:	7 L/min
Collision energy:	10-30V
VCap:	3000 V
Nozzle Voltage:	2000V
Fragmentor:	100 V
Skimmer:	65 V
OCT 1 RF Vpp:	750V
Data Acquisition:	Mass Hunter

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances Difenoconazole in plant protection product GLOB1911F

	Difenoconazole
Author(s), year	Sowle J., 2020a
Principle of method	HPLC-DAD

	Difenoconazole
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity was determined by 16 injections of 8 concentrations of standard ranging from a blank to 1.00 mg/mL. The correlation coefficient was calculated as 1.0000. The linear regression equation is: y = 0.000120x-0.000483.
Precision – Repeatability Mean n = 6 (%RSD)	Six samples of approximately 0.1g of formulated material were prepared in 100mL volumetric flasks. The samples were partially made to volume with Methanol, sonicated for 5 minutes, allowed to cool to room temperature before being made up volume with Methanol. The samples were then injected into the HPLC-DAD. The method is repeatable. The values ranged from 519.80g/L to 524.97g/L with a mean of 523.54g/L, a standard deviation of 1.973 and a percentage relative standard deviation of 0.377.
Accuracy n = 6 (% Recovery)	Formulation blank was spiked using active substance standard solution up to the level present in the formulation. The method is accurate with values of percentage recovery ranging from 100.1% to 101.7%, a mean of 100.9% and a standard deviation of 0.579.
LOQ recovery	LOQ is set at 1g/L. The percentage recovery ranged from 96.89% to 105.5% with a mean of 100.0% and a standard deviation of 2.895.
Interference/ Specificity	There were no analyte interferences. In the specificity chromatograms Difenoconazole Isomers eluted at 10.6 minutes and 11.4 minutes, and other possible significant peaks were accounted for by assaying a solvent blank, a sample of the formulation blank and impurity 1 (toluene) standard. There were no other peaks present in these chromatograms at the same elution time as Difenoconazole Isomers. UV spectral analysis: A Difenoconazole reference standard and the sample were prepared in Methanol and analysed with the same analytical conditions on the HPLC-DAD The Difenoconazole reference standard gave a peak at 10.5 minutes (Isomer 1) and 11.2 minutes (Isomer 2) with a spectral maxima at 205nm, a secondary maxima at 230nm and a tertiary maxima at 275nm, reducing to extinction by 300nm. The sample gave a peak at 10.5 minutes (Isomer 1) and 11.0 minutes (Isomer 2) with a spectral maxima at 205nm a secondary maxima at 230nm and a tertiary maxima at 275nm, reducing to extinction by 300nm, in a similar manner to the Difenoconazole reference standard. The method is specific to Difenoconazole and the spectra produced by both the certified Difenoconazole reference standard and the sample are the same. MS spectral analysis: A Difenoconazole reference standard and the sample were prepared in Methanol and analysed with the same analytical conditions on the LC-QToF. The Difenoconazole reference standard gave a peak at 10.9 minutes (Isomer 1) and 11.7 (Isomer 2) showing the molecular ion of $[M+H]^+$ at 406m/z, with additional ions present at 337m/z, 291m/z, 251m/z and 188m/z. The sample gave a peak at 10.9 minutes (Isomer 1) and 11.7 (Isomer 2) showing the molecular ion of $[M+H]^+$ at 406m/z, with additional ions present at 337m/z, 291m/z, 251m/z and 188m/z, in a similar manner to the Difenoconazole reference standard. The method is specific to Difenoconazole and the spectra produced by both the certified Difenoconazole reference standard and the sample are the same.
Comment	/

Conclusion

The method is suitable for determination of a.s. content Difenoconazole in formulation GLOB1911F and is fully validated according to the SANCO/3030/99 rev.5.

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

An analytical method has been developed for the determination of the impurity Toluene in GLOB1911F.

The following analytical method for the determination of Toluene in the plant protection product GLOB1911F has not previously been reviewed according to the Uniform Principles and is provided in support of this assessment.

Comments of zRMS:	The method is accepted. It meets SANCO/3030/99 rev.5 requirements and may be used for analysing toluene in the PPP.

Reference:	KCP 5.1.1
Report	Validation of the methods of determination of Difenoconazole and a specified impurity in a suspension concentrate formulation GLOB1911F containing 500 g/L Difenoconazole, in compliance with good laboratory practice, Sowle J., DNA5105, 2020a
Guideline(s):	Sanco/3030/99 rev. 5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The assay of Impurity 1 (Toluene) was performed using approximately 0.1g of Formulation. The mass of the formulation was accurately recorded, transferred to a 100 mL volumetric flask and partially made to volume with Methanol. The sample was sonicated for 5 minutes, allowed to cool to room temperature, then made up to volume with methanol. The samples were then assayed by injecting each solution once into the HPLC-DAD under the following conditions:

<u>HPLC-DAD -Impurity 1:</u>

Instrument:	Agilent 1100 Series HPLC-DAD
Mode:	Isocratic Reverse Phase
Column:	Grace Alltima C8, 250 mm x 4.6 mm
Packing:	C8, 5 µm
Eluent:	73% Methanol, 27% Deionised Water adjusted to pH3 with Phosphoric Acid
Wavelength:	210 nm
Flow Rate:	1.0 mL/min
Injection Volume:	5 µL
Column temperature:	25°C
Data collection	Chemstation
Retention Times:	approximately 5.8 to 5.9 minutes

GC-MS conditions – MS Spectral analysis of Impurity 1 (Toluene):

GC-MS Conditions:

Instrument:	Shimadzu GC-MSD with HS-20 Headspace Sampler
Column:	Rtx-1 (30m x 0.32 mm x 0.5µm)
Temperatures column:	60°C for 1 min., then 12°C/min. to 250°C held for 5 min.
Temperatures injector:	25.7°C
Carrier gas:	Helium
Detector:	Scan: 25-250m/z for MS spectral analysis
Data collection:	GCMS Solutions
Retention time:	Appr. 6.5 minutes

Headspace conditions:

Cycle time:	21.83 minutes
Shake Strength:	4/5

Oven Temperature:	70°C
Loop Temperature:	150°C
Transfer line:	180°C

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) GLOB1911F

	Impurity 1 (Toluene)
Author(s), year	Sowle J., 2020a
Principle of method	HPLC-DAD
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity was determined by 18 injections of 9 concentrations of standard ranging from a blank to 0.20 mg/mL. The correlation coefficient was calculated as 1.0000. The linear regresssion equation is y=0.000082x-0.000112.
Precision – Repeatability Mean n = 6 (%RSD)	Six samples of approximately 0.1g of formulated material were prepared in 100mL volumetric flasks. The samples were partially made to volume with Methanol, sonicated for 5 minutes, allowed to cool to room temperature before being made up volume with Methanol. The samples were then injected into the HPLC-DAD. No detectable Impurity 1 above the LOQ level of 0.5 g/kg was detected in the sample.
Accuracy n = 6 (% Recovery)	It is known that the sample (DNA5103/1) contains no detectable toluene above the LOQ Level of 0.5g/Kg. Therefore a Recovery Precision was performed at 5g/Kg. This equates to 0.005mg/ml as the samples were made at l.OOmg/ml concentration. Therefore the Recovery samples were prepared for analysis by spiking samples of DNA5103/1 at 0.005mg/ml using the certified reference standard material. This was achieved by weighing approximately 0.05g of DNA5103/1 into a 50ml volumetric flask, spiked with 1.25ml of 0.2mg/ml Impurity 1 reference standard solution. The samples were partially made to volume with Methanol, sonicated for 5 minutes, allowed cool to room temperature before being made up to volume with Methanol. Six separate solutions were prepared in this way and then injected into the HPLC-DAD. The method is accurate with values of percentage recovery ranging from

	Impurity 1 (Toluene)
	98.79% to 101.3%, a mean of 100.3% and a standard deviation of 1.013 and a percentage relative standaard deviation of 1.010.
LOQ recovery	The LOQ is set at the lowest point of linearity – 0.0005 mg/ml. It corresponds to 0.5 g/kg The percentage recovery ranged from 102.2% to 118.3% with a mean of 111.1% and a standard deviation of 6.923.
Interference/ Specificity	There were no analyte interferences. In the specificity chromatograms the Impurity 1 eluted at 5.8 minutes, and other possible significant peaks were accounted for by assaying a solvent blank, a sample of the formulation blank and reference standard for Difenoconazole. There were no other peaks present in these chromatograms at the same elution time as Impurity 1. UV spectral analysis: An Impurity 1 reference standard and spiked sample were prepared in Methanol and analysed with the same analytical conditions on the HPLC-DAD The Impurity 1 reference standard gave a peak at 5.8 minutes with a spectral maxima at 210nm reducing to extinction by 230nm. The spiked sample gave a Impurity 1 peak at 5.8 minutes with a spectral maxima at 210nm reducing to extinction by 230nm, in a similar manner to the reference standard. The method is specific to Impurity 1 and the spectra produced by both the certified Impurity 1 reference standard and the spiked sample are the same. MS spectral analysis: An Impurity 1 reference standard and a spiked sample were prepared in deionized water containing 40g/L Sodium Chloride and analysed with the same analytical conditions on the GC-MSD with Headspace Sampler. The Impurity 1 reference standard gave a peak at 6.5 minutes showing the primary ion at 92m/z with additional ions at 39m/z, 51 m/z, 65 m/z, and 91 m/z. this was confirmed as Toluen using the NIST11 Database. The spiked sample gave a peak at 6.5 minutes showing the primary ion at 92m/z with additional ions at 39m/z, 51 m/z, 65 m/z, and 91 m/z, in a similar manner to the reference standard. The method is specific to Impurity 1 and the spectra produced by both the certified Impurity 1 reference standard and the spiked sample are the same.
Comment	/

Conclusion

The method is suitable for determination of impurity Toluene in formulation GLOB1911F and is fully validated according to the SANCO/3030/99 rev.5.

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

Under current EU legislation, methods on formulants are not required. However, if a formulant is defined as relevant for toxicity (environment, health), then a method needs to be provided. There are however no formulants in GLOB1911F that are defined as relevant for toxicity.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are no CIPAC methods available for the determination of Difenoconazole.

5.2.2 Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of Difenoconazole for the generation of pre-authorization data is given in the following table.

The below summarized analytical methods for determination of residues in crops were provided in the EU review of Difenoconazole. However, the proposed GAP of GLOB1911F was not the representative GAP in the EU review of Difenoconazole. Therefore residue studies on potatoes, sugar beet and oilseed rape covering the proposed GAP of GLOB1911F were carried out (reference is made to dRR Part B Section 7) and data on the analytical method validation for Difenoconazole in these commodities have also been provided. For these methods reference is made to section 5.3 about post-registration methods.

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

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Component of residue definition: Difenoconazole				
Plants, plant products,... (Residues)	Primary	0.02 mg/kg (apple, lettuce)	LC-MS/MS	SE, 2006 EFSA, 2011
		0.05 mg/kg (wheat grain, oilseed rape)	LC-MS/MS	SE, 2006 EFSA, 2011
	Confirmatory (if required)	-	-	-
Component of residue definition: Difenoconazole alcohol (CGA205375) expressed as Difenoconazole				
Animal products, food of animal origin,... (Residues)	Primary	0.005 mg/kg (milk)	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg (eggs, muscle, fat, kidney, liver)	LC-MS/MS	SE, 2006 EFSA, 2011
	Confirmatory (if required)	-	-	-
Component of residue definition: Difenoconazole and Difenoconazole alcohol (CGA205375) expressed as Difenoconazole				
Soil (Environmental fate, Efficacy, Ecotoxicology)	Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
	Confirmatory (if required)	-	-	-
Component of residue definition: Difenoconazole				
Water (Environmental fate, Efficacy, Ecotoxicology)	Primary	0.05 µg/L (drinking water) 0.1 µg/L (surface water)	GC-ECD	SE, 2006 EFSA, 2011
	Confirmatory (if required)	-	-	-
Air (Exposure)	Primary	0.99 ng/L	LC-MS/MS	SE, 2006 EFSA, 2011
	Confirmatory	-	-	-

Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
	(if required)			
Body fluids and tissues (Toxicology)	Primary	Not relevant, because active substance is not classified as toxic or highly toxic (T/T+)		
	Confirmatory (if required)			

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

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

The methods already submitted in accordance with the requirements set out in point 5.2.1 apply.

5.3.2 Description of analytical methods for the determination of residues 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	0.01 mg/kg	EFSA, 2017a and 2017b
Plant, high acid content		Not needed (no intended use)	-
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	EFSA, 2017a
Plant, high oil content		0.05 mg/kg	EFSA, 2017a
Plant, difficult matrices (hops, spices, tea)		Not needed (no intended use)	-
Muscle	Difenoconazole	0.01 mg/kg	EFSA, 2017a
Milk		0.005 mg/kg	EFSA, 2017a
Eggs		0.01 mg/kg	EFSA, 2017a
Fat		0.01 mg/kg	EFSA, 2017a
Liver, kidney		0.01 mg/kg	EFSA, 2017a

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Soil (Ecotoxicology)	Difenoconazole and Difenoconazole alcohol (CGA205375) expressed as Difenoconazole (data gap needs to be filled before Difenoconazole alcohol (CGA205375) can be excluded, EFSA 2011)	0.01 mg/kg	EFSA, 2011
Drinking water (Human toxicology)	Difenoconazole	0.05 µg/L	EFSA, 2011
Surface water (Ecotoxicology)	Difenoconazole	0.1 µg/L	EFSA, 2011
Air	Difenoconazole	0.99 ng/L	EFSA, 2011
Tissue (meat or liver)	Not required.		
Body fluids			

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 additional studies it is referred to Appendix 2.

An analytical method QuEChERS (CEN/TC 275, 2007-01), using HPLC-MS/MS for the determination of difenoconazole residue (Difenoconazole) in plants has been published, and was considered fully validated with a LOQ = 0.01 mg/kg in dry commodities and matrices with high water content and high acid content. As the QuEChERS method was validated in more than two laboratories for each group, no ILV and no confirmatory method are required (EFSA, 2017a).

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

Component of residue definition: Difenoconazole				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
High water content	Primary	0.02 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	EFSA, 2017a
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011a and 2013a
		0.01 mg/kg	LC-MS/MS	Faessel V., 2012a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011d and 2013b
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006

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**
				EFSA, 2011
		0.01 mg/kg	LC-MS/MS	Austin R., 2009a
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011a and 2013a
		0.01 mg/kg	LC-MS/MS	Faessel V., 2012a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011d and 2013b
High acid content	Primary	0.01 mg/kg	LC-MS/MS	xxxxxx 2008a, 2008b and 2013a
		0.01 mg/kg	LC-MS/MS	EFSA, 2017a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		~~0.01 mg/kg~~	~~LC-MS/MS~~	~~Austin R., 2009a~~
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxx., 2008a, 2008b and 2013a
High oil content	Primary	0.05 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxx., 2008a, 2008b and 2013a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	Austin R., 2009a
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
High protein/high starch content ~~(dry)~~	Primary	0.05 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	EFSA, 2017a
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011a and 2013a
		0.01 mg/kg	LC-MS/MS	Faessel V., 2012a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		~~0.01 mg/kg~~	~~LC-MS/MS~~	~~Austin R., 2009a~~
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
		0.01 mg/kg	LC-MS/MS	Jonchère F., 2011a and 2013a
		0.01 mg/kg	LC-MS/MS	Faessel V., 2012a

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

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	SE, 2006
Not required, because:	/

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

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

Table 5.3-4: Validated methods for food and feed of animal origin (if appropriate)

Component of residue definition: : Difenoconazole alcohol (CGA205375) expressed as Difenoconazole				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Milk	Primary	0.005 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
		0.005 mg/kg	LC-MS/MS	Faessel V., 2013a
	ILV	0.005 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxx., 2009b
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a a
		0.005 mg/kg	LC-MS/MS	Faessel V., 2013a
Eggs	Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxx., 2009b
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
Muscle	Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2009b

Component of residue definition: : Difenoconazole alcohol (CGA205375) expressed as Difenoconazole				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
Fat	Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2009b
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
Kidney, liver	Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a
	ILV	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011
		0.01 mg/kg	LC-MS/MS	xxxxxx., 2009b
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	xxxxxxx., 2008a, 2008b and 2013a

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

Table 5.3-5: Statement on extraction efficiency

	Method for products of animal origin
Required, available from:	SE, 2006
Not required, because:	/

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 additional studies it is referred to Appendix 2.

Table 5.3-6: Validated methods for soil (if appropriate)

Component of residue definition: Difenoconazole and Difenoconazole alcohol (CGA205375) expressed as Difenoconazole			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.01 mg/kg	LC-MS/MS	SE, 2006 EFSA, 2011

Component of residue definition: Difenoconazole and Difenoconazole alcohol (CGA205375) expressed as Difenoconazole			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year / missing**
	0.001 mg/kg	LC-MS/MS	Zietz, E., 2008
Confirmatory			

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

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

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

Table 5.3-7: Validated methods for water (if appropriate)

Component of residue definition: Difenoconazole				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.05 µg/L	GC-ECD	SE, 2006 EFSA, 2011
	ILV			
	Confirmatory	0.05 µg/L	LC-MS/MS	Faessel V., 2013b
Surface water	Primary	0.1 µg/L	GC-ECD	SE, 2006 EFSA, 2011
	Confirmatory	0.1 µg/L	LC-MS/MS	Faessel V., 2013b

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.

Table 5.3-8: Validated methods for air (if appropriate)

Component of residue definition: Difenoconazole			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.99 ng/L	LC-MS/MS	SE, 2006 EFSA, 2011
Confirmatory			

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

Not required as Difenoconazole is not classified as toxic or very toxic.

5.3.2.8 Other studies/ information

In several ecotoxicological studies summarised in section B9 of the dRR analytical methods were used for the detection of the active substance Difenoconazole in the different test mediums. The analytical part of these studies is summarised in Appendix 2.

5.4 References

EFSA (European Food Safety Authority), 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance difenoconazole. EFSA Journal 2011;9(1):1967, 71 pp. https://doi.org/10.2903/j.efsa.2011.1967

EFSA (European Food Safety Authority), 2017a. Reasoned Opinion on the modification of the existing maximum residue levels for difenoconazole in various crops. EFSA Journal 2017;15(7):4893, 33 pp. https://doi.org/10.2903/j.efsa.2017.4893

EFSA (European Food Safety Authority), 2017b. Reasoned opinion on the modification of the existing maximum residue levels for difenoconazole in various crops. EFSA Journal 2018;16(1):5143, 30 pp. doi:10.2903/j.efsa.2018.5143

Sweden, 2006. Draft assessment report on the active substance difenoconazole prepared by the rapporteur Member State Sweden in the framework of Council Directive 91/414/EEC, December 2006.

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
KCP 5.1.1	Sowle J.	2020a	Validation of the methods of determination of Difenoconazole and a specified impurity in a suspension concentrate formulation GLOB1911F containing 500 g/L Difenoconazole, in compliance with good laboratory practice. Study Number: DNA5105 David Norris Analytical Laboratories Ltd. GLP Unpublished	N	Globachem NV
KCP 5.1-05	Gätschenberger H.	2017	Difenoconazole and Paclobutrazol – Residues in honey following exposure of bees to treated winter oilseed rape in Germany during 2016. Study Number: S16-01988 GLP not published	N	Syngenta*
KCP 5.1-06 (submitted as KCP 10.2.1/01)	Juckeland, D.	2020a	Effects of GLOB1911F on *Pseudokirchneriella subcapitata* in an algal growth inhibition test BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 48 AAL 0002 GLP Unpublished	N	Globachem NV
KCP 5.1-07 (submitted as 10.6.2/01)	Kästner K.,	2020a	Effect of GLOB1911F on seedling emergence and seedling growth of six non-target terrestrial plant species under greenhouse conditions BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 46 PSE 0002 GLP Unpublished	N	Globachem NV
KCP 5.1-08 (submitted as	Kästner K.,	2020b	Effect of GLOB1911F on vegetative vigour of six non-target terrestrial plant species under greenhouse conditions	N	Globachem NV

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
10.6.2/02)			BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 46 PVV 0002 GLP Unpublished		
KCP 5.1-09 (Submitted as KCP 10.3.1.1	Amsel K.,	2020	Acute toxicity of GLOB1911F to the bumblebee *Bombus terrestris L.* under laboratory conditions. BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 48 BBA 0010 GLP Unpublished	N	Globachem NV
KCP 5.1-10 (Submitted as KCA 8.2.5.3/01)	Eckenstein H.,	2014	Difenoconazole - Effects on the Development of Sediment-Dwelling Larvae of Chironomus riparius in Water-Sediment Systems with Spiked Sediment Harlan Laboratories Ltd, Zelgliweg 1, 4452 Itingen, Switzerland Report Number D81747. Syngenta File No. CGA169374_10839 GLP Unpublished	N	Syngenta*
KCP 5.1-11	Dreßler K.,	2020	Chronic toxicity of GLOB1911F to the honey bee *Apis mellifera L.* under laboratory conditions Verification of the concentration of difenoconazole in feeding solutions by LC-MS/MS BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 48 BAC 0003 Analytical report: 20 35 CRB 0005 GLP Unpublished	N	Globachem NV
KCP 5.1-12	Schmidt K.,	2020	GLOB1911F – Repeated exposure of honey bee (Apis mellifera L.) larvae under laboratory conditions Verification of the concentration of difenoconazole in test item stock solutions by LC-MS/MS BioChem agrar, Labor für biologische und chemische Analytik GmbH Report number 20 48 BLC 0004 Analytical report: 20 35 CRB 0006 GLP Unpublished	N	Globachem NV

*Globachem N.V. has a Letter of Access to this study from Syngenta.

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	Verte-brate study Y/N	Owner
KCP 5.1-01a	Jonchère F.	2011a	Validation of the Analytical Method for the Determination of Difenoconazole Residues in potato (tubers) and wheat (grain and straw) Laboratory: Anadiag Study number: R B0128 GLP Unpublished	N	Globachem NV
KCP 5.1-01b	Jonchère F.	2013a	Amendment No. 1 to final Report Number R B0128: Validation of the Analytical Method for the Determination of Difenoconazole Residues in potato (tubers) and wheat (grain and straw) Laboratory: Anadiag Study number: R B0128 GLP Unpublished	N	Globachem NV
KCP 5.1-02	Faessel V.	2012a	Validation of the analytical method for the determination of Difenoconazole residue in Apricot, sugar beet (roots and leaves), carrot (roots) and celery (leaves) Laboratory: Anadiag Study number: R B2196 GLP Unpublished	N	Globachem NV
KCP 5.1-03a	xxxxxx	2008a	Validation of the Analytical Methods for the Determination of Difenoconazole (and its metabolites) Residues in vegetables, fruits, cereals and animal matrices Laboratory: xxxxxx Study number: R A8143 GLP Unpublished	N	Globachem NV
KCP 5.1-03b	xxxxxx	2008b	Amendment No. 1 to final Report Number R A8143: Validation of the Analytical Methods for the Determination of Difenoconazole (and its metabolites) Residues in vegetables, fruits, cereals and animal matrices Laboratory: xxxxx	N	Globachem NV

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Study number: R A8143 GLP Unpublished		
KCP 5.1-03c	xxxxxx	2013a	Amendment No. 2 to final Report Number R A8143: Validation of the Analytical Methods for the Determination of Difenoconazole (and its metabolites) Residues in vegetables, fruits, cereals and animal matrices Laboratory: xxxxxx Study number: R A8143 GLP Unpublished	N	Globachem NV
KCP 5.1-04a (submitted as KCA 6.3-07a)	Jonchère F.	2011d	Determination of Difenoconazole residues in oilseed rape following treatment with Difenoconazole 250 EC under field conditions in Northern and Southern Europe in 2011. Lab: Anadiag SA, France Study Number: R B1114 GLP not published	N	Globachem NV
KCP 5.1-04b (submitted as KCA 6.3-07b)	Jonchère F.	2013b	Amendment No. 1 to final Report Number R B1114: Determination of Difenoconazole residues in oilseed rape following treatment with Difenoconazole 250 EC under field conditions in Northern and Southern Europe in 2011. Lab: Anadiag SA, France Study Number: R B1114 GLP not published	N	Globachem NV
KCP 5.2-01	Austin R.	2009a	Independent Laboratory Validation of a Method for the determination of difenoconazole in crops Laboratory: CEM Analytical Service (CEMAS) Study number: CEMS-4180 GLP Unpublished	N	Globachem NV
KCP 5.2-02	xxxxxx	2009b	Independent Laboratory Validation of a Method for the determination of difenoconazole and its metabolite CGA205375 in bovine liver, kidney, muscle and fat, milk and eggs	N	Globachem NV

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Verte-brate study Y/N	Owner
			Laboratory: xxxxxxxx Study number: CEMS-4181 GLP Unpublished		
KCP 5.2-03	Faessel V.	2013a	Validation of the Analytical Method for the Determination of Difenoconazole Residues in milk. Lab: Anadiag SA, France Study Number: R B2295 GLP not published	N	Globachem NV
KCP 5.2-04	Zietz E.	2008	Validation of an Analytical Method for the Determination of Difenoconazole and its Metabolite CGA 205375 in Soil Laboratory: SGS INSTITUT FRESENIUS GmbH Study number: IF-08/01135942 GLP Unpublished	N	Globachem NV
KCP 5.2-05	Faessel V.	2013b	Validation of the Analytical Method for the Determination of Difenoconazole Residues in ground and surface water. Lab: Anadiag SA, France Study Number: R B2299 GLP not published	N	Globachem NV

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for Difenoconazole

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

Reference is made to sections 5.2.1.1 and 5.2.1.2 in addition to the summaries below.

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

A 2.1.1.1.1 Analytical method 1 – LC/MS-MS

This study (Jonchère F., 2013a) has already been submitted in Poland (zRMS) to support the authorisation of the registered product Narita and was positively evaluated. For completeness, the summary is given below once again.

A 2.1.1.1.1.1 Method validation

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 0.7.03.2016 prepared by Eko-Futura Sp. z.0.0; IOR for the extension of authorisation of product Difcor 250 EC for minor uses. The following conclusion was made: "*To be fit for the intended purpose, the method was successfully evaluated and met certain validation characteristics: specificity, linearity, repeatability and accuracy. The analytical method fulfils the criteria of SANCO/825/00 rev. 8.1.* *The study has been performed in compliance with Good Laboratory Practice and is acceptable."*

Reference:	KCP 5.1-01
Report	Validation of the Analytical Method for the Determination of Difenoconazole Residues in potato (tubers) and wheat (grain and straw), Jonchère F., 2011a, R B0128 Amendment No. 1 to final Report Number R B0128, Jonchère F., 2013a
Guideline(s):	Yes (SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method was validated for difenoconazole in potato (tubers) and wheat (grain and straw). The principle of the method for difenoconazole is based on a manual extraction of the crop with acetone,

followed by a clean-up of the crude extract by a liquid/liquid partition with dichloromethane. The residues are dissolved in hexane/ethanol.

Before analysis of wheat straw, the internal standard triphenyl phosphate was added to the extract. Difenoconazole is then quantified by UPLC with MS/MS detection with the following conditions:

UPLC-MS/MS conditions:

Analytical Conditions
LC-MS/MS
for potato tubers and grain analysis
No. MA_616-01

Apparatus	LC /MS /MS

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1*100 mm	Supplier reference	186002352	Temperature	40 °C
Development Column ANADIAG Number	130	Stationary Phase	C18	Comment	-

Mobile phase

A =	H_2O MilliQ + 0.1 % formic acid	C =	-
B =	Methanol + 0.1 % formic acid	D =	-

Sample temperature	15 ° C

Elution

Elution	*Time*	*Flow*	Composition (%)				*Curve*	Elution	*Time*	*Flow*	Composition (%)				*Curve*
	min	mL/min	A	B	C	D	(type)		min	mL/min	A	B	C	D	(type)
Pg1	0.00	0.4	90	10	-	-	0	Pg5	8.00	0.4	90	10	-	-	6
Pg2	6.00	0.4	0	100	-	-	6	Pg6	-	-	-	-	-	-	-
Pg3	6.50	0.4	0	100	-	-	6	Pg7	-	-	-	-	-	-	-
Pg4	6.90	0.4	90	10	-	-	6	Pg8	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES x	APCI
Polarity*	Pos x	Neg

*cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1*	TRANSITION 2**	TRANSITION 3	RT (min.)
				Parent > Daughter	Parent > Daughter	Parent > Daughter	
difenoconazole	35	20	50	406.4>251.3	-	-	5.78
	35	16	50	-	406.4>337.2	-	5.78

* quantifier transition
** qualifier transition

Date of application of analytical conditions: 28/07/10

Study	B0128	Column ANADIAG number	169
Matrix	Potato Tubers Wheat grain	Retention time	Difenoconazole: ≈ 5.7 min.
Sample temperature	+15 °C	Injected volume	10 µL

Analytical Conditions
LC-MS/MS
for straw analysis
No. MA_616-02

Apparatus	**LC /MS /MS**

Column

Description	BEH C18	**Supplier**	WATERS	**Particles**	1.7 µm
Internal diam. x length	2.1*100 mm	**Supplier reference**	186002352	**Temperature**	40 °C
Development Column ANADIAG Number	130	**Stationary Phase**	C18	**Comment**	-

Mobile phase

A =	H_2O MilliQ + 0.1 % formic acid	**C =**	-
B =	Methanol + 0.1 % formic acid	**D =**	-

Sample temperature	15 ° C

Elution

Elution	*Time*	*Flow*	Composition (%)				*Curve*	Elution	*Time*	*Flow*	Composition (%)				*Curve*
	min	mL/min	A	B	C	D	(type)		min	mL/min	A	B	C	D	(type)
Pg1	0.00	0.4	90	10	-	-	0	**Pg5**	8.00	0.4	90	10	-	-	6
Pg2	6.00	0.4	0	100	-	-	6	**Pg6**	-	-	-	-	-	-	-
Pg3	6.50	0.4	0	100	-	-	6	**Pg7**	-	-	-	-	-	-	-
Pg4	6.90	0.4	90	10	-	-	6	**Pg8**	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES **x**	APCI
Polarity*	Pos **x**	Neg

*cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1* Parent > Daughter	TRANSITION 2* Parent > Daughter	TRANSITION 3 Parent > Daughter	RT (min.)
difenoconazole	35	20	50	406.4>251.3	-	-	5.78
	35	16	50	-	406.4>337.2	-	5.78
TPP	40	27	50	327>215	-	-	5.59

* quantifier transition
** qualifier transition

Date of application of analytical conditions: 05/08/10

Study	B0128	**Column ANADIAG number**	169
Matrix	Wheat Straw	**Retention time**	Difenoconazole: ≈ 5.7 min. TTP: ≈ 5.50 min.
Sample temperature	+15 °C	**Injected volume**	10 µL

Results and discussions

Validation data: *Specificity/ interferences :*	Chromatograms of standards solution, of matrix fortified and of control specimen of potatoes and wheat grain are given in the dossier. No interferences are observed at the retention time of difenoconazole. Amendment No. 1 to final report R B0128: Data available compliant to the specificity demonstration according to SANCO/825/00 rev. 8.1 by monitoring one additional transition. The method is considered as highly specific.
Linearity :	For wheat grain and potato tubers, the detector response for difenoconazole analysis was linear within the range from 1.7 ng/mL to 60.2 ng/mL with $r^2 > 0.999$ (n=7) (y = -1.95 + 0.0050785 x). For wheat straw, the detector response for difenoconazole analysis was linear within the range from 1.7 ng/mL to 60.2 ng/mL with $r^2 > 0.99$ (n=7) (y = -0.0086903 + 0.37304 x).

Accuracy / repeatability :	The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation:

Analyte	Matrix	Fortification level (mg/kg)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	Potato tubers	0.01	75.2%	1.1%	1.5%	5
		0.1	91.4%	12.5%	13.6%	5
		All levels	83.3%	11.9%	14.3%	10
Difenoconazole	Wheat grain	0.01	76.8%	4.2%	5.5%	5
		0.1	81.3%	8.5%	10.5%	5
		All levels	79.1%	6.7%	8.5%	10
Difenoconazole	Wheat straw	0.01	87.0%	11.9%	13.6%	5
		0.1	88.7%	11.5%	12.9%	5
		All levels	87.9%	11.0%	12.5%	10

The accuracy of the method is acceptable

LOQ	The LOQ of the method is 0.01mg/kg.

Conclusion

The method is suitable for determination of difenoconazole in potato/tubers, wheat/grain and straw. The method is considered as highly specific.

A 2.1.1.1.2 Analytical method 2 – LC/MS-MS

This study (Faessel V., 2012a) has already been submitted in Poland (zRMS) to support the authorisation of the registered product Difcor 250 EC and was positively evaluated. For completeness, the summary is given below once again.

A 2.1.1.1.2.1 Method validation

Comments of zRMS:	The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 0.7.03.2016 prepared by Eko-Futura Sp. z.0.0; IOR for the extension of authorisation of product Difcor 250 EC for minor uses. The following conclusion was made: *"To be fit for the intended purpose, the method was successfully evaluated and met certain validation characteristics: specificity, linearity, repeatability and accuracy. The analytical method fulfils the criteria of SANCO/825/00 rev. 8.1. The study has been performed in compliance with Good Laboratory Practice and is acceptable."*

Reference:	KCP 5.1-02
Report	Validation of the analytical method for the determination of Difenoconazole residue in Apricot, sugar beet (roots and leaves), carrot (roots) and celery (leaves), Faessel V., 2012a, R B2196
Guideline(s):	Yes (SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4)
Deviations:	No

GLP:	Yes
Acceptability:	Yes

Materials and methods

The method was validated for difenoconazole in apricot, sugar beet (roots and leaves), carrot (roots) and celery (leaves).
The principle of the method for difenoconazole is based on a manual extraction of the crop with acetone, followed by a clean-up of the crude extract by a liquid/liquid partition with dichloromethane. The residues are dissolved in hexane/ethanol and then quantified by UPLC with MS/MS.

UPLC-MS/MS conditions:

Analytical Conditions
LC-MS/MS

No. MA_616-04

Apparatus	WATERS XEVO TQ-MS UPLC/MS/MS

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1*50 mm	Supplier reference	186002350	Temperature	40 °C
Development Column ANADIAG Number	168	Stationary Phase	C18	Comment	-

Mobile phase

A =	H_2O MilliQ + 0.1 % formic acid	C =	-
B =	Methanol + 0.1 % formic acid	D =	-

Sample temperature	15 ° C

Elution

Elution	*Time* min	*Flow* mL/min	Composition (%) A	B	C	D	*Curve* (type)	Elution	*Time* min	*Flow* mL/min	Composition (%) A	B	C	D	*Curve* (type)
Pg1	0.00	0.4	90	10	-	-	0	Pg5	4.00	0.4	90	10	-	-	6
Pg2	2.00	0.4	0	100	-	-	6	Pg6	-	-	-	-	-	-	-
Pg3	3.20	0.4	0	100	-	-	6	Pg7	-	-	-	-	-	-	-
Pg4	3.25	0.4	90	10	-	-	6	Pg8	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES x	APCI
Polarity*	Pos x	Neg

*make a cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1 Parent > Daughter	TRANSITION 2 Parent > Daughter	RT (min.)
DIFENOCONAZOLE	35	20	50	406.4>251.3	-	2.7
	35	16	50	-	406.4>337.2	

Date of application of analytical conditions: 30/07/2012

Study	B2196	Column ANADIAG number	168
Matrix	Apricot, sugar beet (roots and leaves), carrot (roots) and celery (leaves)	Retention time	Difenoconazole: ≈ 2.7 min.
Sample temperature	+15 °C	Injected volume	10 µL

Results and discussions

Validation data:	
Specificity/ interferences :	Chromatograms of standards solution, of matrix fortified and of control specimen are given for Difenoconazole No interferences are observed at the retention time of difenoconazole The method is considered as specific.
Linearity :	For both quantifier and qualifier ions, the detector response for difenoconazole analysis was linear within the range from 1.5 ng/mL to 62.7 ng/mL with $r^2 > 0.99$ ($y = 0.26 + 0.0015674 x$ for the quantifier and $y = 0.59 + 0.0049202 x$ for the qualifier) ($n>5$).

Accuracy / repeatability :	The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation: Quantification:

Analyte	Matrix	Fortification level (mg/kg)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	Apricot	0.01	84.4%	8.1%	9.7%	5
		0.10	84.0%	5.5%	6.5%	5
		All levels	84.2%	6.5%	7.8%	10
	Sugar beet roots	0.01	97.9%	5.5%	5.6%	5
		0.10	77.9%	5.1%	6.5%	5
		All levels	87.9%	11.6%	13.2%	10
	Sugar beet leaves	0.01	100.6%	5.5%	5.4%	5
		0.10	89.3%	4.6%	5.2%	5
		All levels	95.0%	7.7%	8.1%	10
	Carrot (roots)	0.01	75.7%	3.0%	3.9%	5
		0.10	99.3%	8.3%	8.3%	5
		All levels	87.5%	13.7%	15.7%	10
	Celery (leaves)	0.01	95.3%	4.3%	4.5%	5
		0.10	92.6%	5.4%	5.8%	5
		All levels	93.9%	4.8%	5.1%	10

Confirmation:

Analyte	Matrix	Fortification level (mg/kg)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	Apricot	0.01	84.8%	7.4%	8.8%	5
	Sugar beet roots	0.01	102.6%	6.7%	6.5%	5
	Sugar beet leaves	0.01	98.1%	6.3%	6.4%	5
	Carrot (roots)	0.01	74.5%	3.2%	4.3%	5
	Celery (leaves)	0.01	85.1%	8.7%	10.2%	5

The Accuracy of the method is acceptable.

LOQ	The LOQ of the method for the determination of the Difenoconazole is 0.01mg/kg.

Conclusion

The method is suitable for determination of difenoconazole by LC-MS/MS at the LOQ=0.01mg/kg. The method is considered as specific.

A 2.1.1.1.3 Analytical method 3 – LC/MS-MS

This study (xxxxxx, 2008b) has already been submitted in Poland (zRMS) to support the authorisation of the registered products Difcor 250 EC and Narita and was positively evaluated. For completeness, the summary is given below once again.

A 2.1.1.1.3.1 Method validation

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. This study was previously positively evaluated in Part B Section 2 of the Registration Report from 18.01.2014 prepared by Eko-Futura Sp. z o.o., IOR for the Step 2 dossier of Difcor 250 EC. The following conclusion was made (cited by

	translation): "The presented method meets the required criteria to ensure the correct determination of the residue of difenoconazole and its metabolite CGA205375 in seeds of winter rape, apples and pears and in food of animal origin. *The obtained results for individual components meet the requirements of SANCO / 825/00 rev.7 and SANCO / 3029/99 rev.4 on 11 July 2000. The presented test method is suitable for use in accordance with the application table of Difcor 250 EC in selected central zone countries."*

Reference:	KCP 5.1-03
Report	Validation of the Analytical Methods for the Determination of Difenoconazole (and its metabolites) Residues in vegetables, fruits, cereals and animal matrices, xxxxx. 2008a, R A8143 Amendment No. 1 to final Report Number R A8143, xxxxxx 2008b Amendment No. 2 to final Report Number R A8143, xxxxxx., 2013a
Guideline(s):	Yes (SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method was validated for difenoconazole in apple/fruit, carrots/roots, oilseed rape/seeds and grapes/berry as well as for difenoconazole and CGA205375 in milk, eggs, fat from meat, liver, kidney and bovine muscle.

The principle of the method is based on a manual or automatic extraction of the crop with acetone, followed by a clean-up of the crude extract by a liquid/liquid partition with dichloromethane. The residues are dissolved in hexane/ethanol and then quantified by UPLC-MS/MS detection with the following conditions:

UPLC-MS/MS conditions:

Apparatus	LC /MS /MS

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1*100 mm	Supplier reference	186002352	Temperature	40 °C
Development Column ANADIAG Number	130	Stationary Phase	C18	Comment	-

Mobile phase

A =	H_2O MilliQ + 0.1 % formic acid	C =	-
B =	Methanol + 0.1 % formic acid	D =	-

Sample temperature	15 ° C

Elution

Elution	*Time* min	*Flow* mL/min	Composition (%) A	B	C	D	*Curve* (type)	Elution	*Time* min	*Flow* mL/min	Composition (%) A	B	C	D	*Curve* (type)
Pg1	0.00	0.4	90	10	-	-	0	Pg5	8.00	0.4	90	10	-	-	6
Pg2	6.00	0.4	0	100	-	-	6	Pg6	-	-	-	-	-	-	-
Pg3	6.50	0.4	0	100	-	-	6	Pg7	-	-	-	-	-	-	-
Pg4	6.90	0.4	90	10	-	-	6	Pg8	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES x	APCI
Polarity*	Pos x	Neg

*make a cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1 Parent > Daughter	TRANSITION 2 Parent > Daughter	TRANSITION 3 Parent > Daughter	RT (min.)
DIFENOCONAZOLE	35	25	50	405.6>250.8	-	-	5.78
	35	15	50	-	405.6>336.8	-	5.78
CGA 205375	30	15	50	349.8>70.0	-	-	5.39
	30	15	50	-	351.8>70.0	351.8>282.8	5.39

Date of application of analytical conditions

Study	A8143	Column ANADIAG number	130
Matrix	various	Retention time	Difenoconazole: ≈ 5.78 min. CGA205375: ≈ 5.39 min.
Sample temperature	+15 °C	Injected volume	10 µL

Results and discussions

Validation data:	
Specificity/ interferences :	Chromatograms of standards solution, of matrix fortified and of control samples (apple, carrots, oilseed rape, grapes fruit, milk, eggs, fat, liver, kidney and muscle) are given in the dossier. No interferences are observed at the retention time of difenoconazole and CGA205375. Amendment No. 2 to final report R A8143: Data available compliant to the specificity demonstration according to SANCO/825/00 rev. 8.1 by monitoring one additional transition. The method is considered as highly specific.
Linearity :	The detector response for **Błąd! Nie można odnaleźć źródła odwołania.** and CGA205375 analysis was linear within the range from 1.6 ng/mL to 60 ng/mL (n=7) with $r^2 > 0.999$ (y = -0.06 + 0.0023286 x for difenoconazole and y = 0.03 + 0.0053226 x for CGA205375).
Accuracy / repeatability :	The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation:

Matrix	Extraction	Active ingredient	Fortification level (mg/kg)	Percentage of mean recovery (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Apple	Automatic	Difenoconazole	0.01 mg/kg	73.2%	1.4%	1.9%	5
			0.10 mg/kg	79.8%	5.3%	6.6%	5
			All levels	76.5%	5.0%	6.6%	10
	Manual		0.01 mg/kg	98.7%	9.5%	9.6%	3
			0.10 mg/kg	92.9%	7.4%	7.9%	3
			All levels	95.8%	8.2%	8.6%	6
Carrot (roots)	Automatic	Difenoconazole	0.01 mg/kg	73.3%	0.8%	1.1%	5
			0.10 mg/kg	81.0%	4.7%	5.7%	5
			All levels	77.2%	5.2%	6.7%	10
	Manual		0.01 mg/kg	81.5%	2.6%	3.2%	3
			0.10 mg/kg	82.4%	9.4%	11.4%	3
			All levels	81.9%	6.2%	7.6%	6
Oilseed rape (seeds)	Automatic	Difenoconazole	0.01 mg/kg	82.9%	9.3%	11.2%	5
			0.10 mg/kg	74.4%	2.5%	3.3%	5
			All levels	78.6%	7.8%	9.9%	10
	Manual		0.01 mg/kg	85.3%	12.5%	14.6%	3
			0.10 mg/kg	94.0%	3.6%	3.9%	3
			All levels	89.7%	9.5%	10.6%	6
Grapes	Manual	Difenoconazole	0.01 mg/kg	75.3%	2.3%	3.1%	5
			0.10 mg/kg	88.8%	6.3%	7.0%	5
			All levels	82.0%	8.4%	10.2%	10

Matrix	Active ingredient	Extraction	Fortification level (mg/kg)	Percentage of mean recovery (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Milk	Difenoconazole	Manual	0.01 mg/kg	103.0%	2.6%	2.6%	5
			0.10 mg/kg	96.1%	2.2%	2.3%	5
			All levels	99.5%	4.3%	4.3%	10
	CGA205375	Manual	0.01 mg/kg	99.3%	4.7%	4.8%	5
			0.10 mg/kg	96.8%	1.6%	1.6%	5
			All levels	98.1%	3.6%	3.6%	10
Eggs	Difenoconazole	Manual	0.01 mg/kg	97.0%	2.5%	2.6%	5
			0.10 mg/kg	107.6%	4.9%	4.6%	5
			All levels	102.3%	6.7%	6.6%	10
	CGA205375	Manual	0.01 mg/kg	90.1%	4.3%	4.8%	5
			0.10 mg/kg	100.9%	5.4%	5.4%	5
			All levels	95.5%	7.3%	7.7%	10
Fat from meat	Difenoconazole	Manual	0.01 mg/kg	74.5%	3.3%	4.4%	5
			0.10 mg/kg	72.2%	2.7%	3.8%	5
			All levels	73.4%	3.1%	4.2%	10
	CGA205375	Manual	0.01 mg/kg	92.7%	1.8%	1.9%	5
			0.10 mg/kg	89.3%	1.6%	1.8%	5
			All levels	91.0%	2.4%	2.6%	10
Liver	Difenoconazole	Manual	0.01 mg/kg	88.9%	3.6%	4.1%	5
			0.10 mg/kg	83.4%	1.9%	2.2%	5
			All levels	86.2%	4.0%	4.6%	10
	CGA205375	Manual	0.01 mg/kg	84.7%	3.4%	4.0%	5
			0.10 mg/kg	84.7%	2.9%	3.4%	5
			All levels	84.7%	3.0%	3.5%	10
Kidney	Difenoconazole	Manual	0.01 mg/kg	87.5%	4.2%	4.8%	5
			0.10 mg/kg	91.6%	5.1%	5.6%	5
			All levels	89.5%	4.9%	5.5%	10
	CGA205375	Manual	0.01 mg/kg	88.0%	4.2%	4.8%	5
			0.10 mg/kg	94.2%	4.4%	4.7%	5
			All levels	91.1%	5.2%	5.7%	10
Bovine muscle	Difenoconazole	Manual	0.01 mg/kg	80.0%	2.2%	2.8%	5
			0.10 mg/kg	81.5%	1.0%	1.2%	5
			All levels	80.7%	1.8%	2.3%	10
	CGA205375	Manual	0.01 mg/kg	91.2%	2.4%	2.6%	5
			0.10 mg/kg	91.7%	0.8%	0.9%	5
			All levels	91.4%	1.7%	1.9%	10

LOQ	The LOQ of the method is 0.01mg/kg

Conclusion

The method is suitable for determination of Difenoconazole in apple/fruit, carrots/roots, oilseed rape/seeds and grapes/berry as well as for Difenoconazole and CGA205375 in milk, eggs, fat from meat, liver, kidney and bovine muscle.

A 2.1.1.1.4 Analytical method 4 – LC/MS-MS

This study (Jonchère F., 2011d) has already been submitted in Poland (zRMS) to support the authorisation of the registered product Difcor 250 EC and was positively evaluated. For completeness, the summary is given below once again.

A 2.1.1.1.4.1 Method validation

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 0.7.03.2016 prepared by Eko-Futura Sp. z.0.0; IOR for the extension of authorisation of product Difcor 250 EC for minor uses. The following conclusion was made: "*To be fit for the intended purpose, the method was successfully evaluated and met certain validation characteristics: specificity, linearity, repeatability and accuracy.* *The method was used for the generation of pre-authorisation data.* *The study has been performed in compliance with Good Laboratory Practice and is acceptable."*

Reference:	KCP 5.1-04 (submitted as KCA 6.3-07)
Report	Determination of Difenoconazole residues in oilseed rape following treatment with Difenoconazole 250 EC under field conditions in Northern and Southern Europe in 2011. Jonchère F., 2011d, R B1114 Amendment No. 1 to final Report Number R B1114, Jonchère F., 2013b
Guideline(s):	Yes (SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method used (xxxxxx., 2008a) for determination of difenoconazole residue in oilseed rape/whole plants /rest of plants was fully validated in the monitoring part. The principle of the method is based on a manual or automatic extraction of the crop with acetone, followed by a clean-up of the crude extract

by a liquid/liquid partition with dichloromethane. The residues are dissolved in hexane/ethanol and then quantified by LC with MS/MS detection.

UPLC-MS/MS conditions:

Analytical Conditions
LC-MS/MS

No. MA_616-01

Apparatus	LC /MS /MS

Column

Description	BEH C18	**Supplier**	WATERS	**Particles**	1.7 µm
Internal diam. x length	2.1*100 mm	**Supplier reference**	186002352	**Temperature**	40 °C
Development Column ANADIAG Number	130	**Stationary Phase**	C18	**Comment**	-

Mobile phase

A =	H2O MilliQ + 0.1 % formic acid	**C =**	-
B =	Methanol + 0.1 % formic acid	**D =**	-

Sample temperature	15 ° C

Elution

Elution	Time	Flow	Composition (%)				Curve	Elution	Time	Flow	Composition (%)				Curve
	min	mL/min	A	B	C	D	(type)		min	mL/min	A	B	C	D	(type)
Pg1	0.00	0.4	90	10	-	-	0	Pg5	8.00	0.4	90	10	-	-	6
Pg2	6.00	0.4	0	100	-	-	6	Pg6	-	-	-	-	-	-	-
Pg3	6.50	0.4	0	100	-	-	6	Pg7	-	-	-	-	-	-	-
Pg4	6.90	0.4	90	10	-	-	6	Pg8	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES x	APCI
Polarity*	Pos x	Neg

*cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1 Parent > Daughter	TRANSITION 2 Parent > Daughter	TRANSITION 3 Parent > Daughter	RT (min.)
DIFENOCONAZOLE	35	25	50	406.1>250.9	-	-	5.78
	35	15	50	-	406.1>337.1	-	5.78
CGA 205375	30	15	50	349.8>70.0	-	-	5.39
	30	15	50	-	351.8>70.0	351.8>282.8	5.39

Date of application of analytical conditions: 30/08/2011

Study	B1114	**Column ANADIAG number**	161
Matrix	Oilseed rape	**Retention time**	Difenoconazole: ≈ 5.7 min.
Sample temperature	+15 °C	**Injected volume**	10 µL

Results and discussions

Validation data:

Specificity/ interferences :

Chromatograms of standards solution, of treated matrix and of control samples are given in the dossier. No interferences are observed at the retention time of difenoconazole.

Amendment No. 1 to final report R B1114:
Data available compliant to the specificity demonstration according to SANCO/825/00 rev. 8.1 by monitoring one additional transition.
The method is considered as highly specific.

Linearity :

The detector response for difenoconazole analysis was linear within the range from 1.6 ng/mL to 60.6 ng/mL with $r^2 > 0.990$ (n= 7) (y = -0.67 + 0.00015249 x for difenoconazole).

Accuracy / repeatability :

The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation:

Analyte	Matrix	Fortification level (mg/kg)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
		0.01	76.4	3.7	4.8	5

Difenoco-nazole	Whole plants / rest of plants	0.10	84.4	3.3	4.0	5

Analyte	Matrix	Fortification level (mg/kg)	recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoco-nazole	Pods	0.1	86.7	-	-	1
	seeds	0.1	78.5	-	-	1
		0.01	75.9/73.9	5.8	7	2
	Rest of plants without roots	0.01	77.0	-	-	1
	Whole plant without root	0.01	77.0/75.3	1.2	1.6	2
		0.1	73.6	-	-	1
		0.256	105.4	-	-	1
		0.513	95.0	-	-	1

The accuracy of the method is acceptable

The frozen storage stability of difenoconazole extracts in oilseed rape seeds, pods and whole plants without roots during frozen storage was investigated. The results indicate a good stability for at least 6 days in seeds, 11 days in pods and 9 days in whole plants without roots (1 sample was tested at a fortification level of 0.1 ppm for whole plant without roots and pods and at 0.01 ppm for seeds) as can be seen in the following table.

Results of the storage stability of difenoconazole extracts in oilseed rape seeds, pods and whole plants without roots

Extraction date	Date of 1st analysis	Recovery 1st analysis (%)	Date of 2nd analysis	Recovery 2nd analysis (%)	Storage time (days)	Matrix
06/09/11	09/09/11	73.9	12/09/11	77.7	6	Seeds
30/08/11	01/09/11	86.7	10/09/11	79.4	11	Pods
01/09/11	03/09/11	73.6	10/09/11	82.0	9	Whole plants without roots

The storage stability of residues in extracts is found to be good as the recovery is in acceptable limit.

Conclusion

The analytical method is considered as validated for the determination of Difenoconazole residue in plant with oil content for residue trials.

A 2.1.1.2 Description of analytical methods of studies used in Part B Section 9 - Eco-toxicology

Comments of zRMS:	The study is acceptable. The method employed in the study has been accepted. The zRMS agrees with the applicant's conclusion written below.

Reference:	KCP 5.1-05
Report	Difenoconazole and Paclobutrazol – Residues in honey following exposure of bees to treated winter oilseed rape in Germany during 2016, Gätschenberger H., 2017, report No S16-01988.
Guideline(s):	EC (1997) Guidance Document 7029/VI/95 rev. 5 SANCO/3029/99 rev. 4 (11/07/2000) SANCO/825/00 rev. 8.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Executive summary

In the analytical phase report of study S16-01988, analytical method GRM066.03A, used in study KCA 6.10.1 in dRR section B7, was validated for the determination of residues of Difenoconazole in honey.

Materials and methods

Samples were extracted under reflux with methanol:concentrated ammonium hydroxide (80/20, v/v). Extracts were allowed to cool and settle. Aliquots were then taken and diluted with ultra-pure water followed by purification by solid phase extraction (SPE).
Difenoconazole was analysed by high performance liquid chromatography (LC-MS/MS).

Results and discussion

Table A 1: Characteristics for the analytical method used for validation of Difenoconazole residues in honey

	Difenoconazole
Recovery	Recovery values for Difenoconazole at the LOQ were in the range 90-108% with a mean recovery of 101% and 7.7% relative standard deviation (RSD) for the primary mass transition (m/z 406/251) and were in te range 88-106% with a mean recovery of 98% and 9.8% relative standard deviation (RSD) for the confirmatory mass transition (m/z 406/188). Recovery values for Difenoconazole at 10xLOQ were in the range 78-93% with a mean recovery of 88% and 6.8% relative standard deviation (RSD) for the primary mass transition (m/z 406/251) and were in te range 79-88% with a mean recovery of 91% and 8.5% relative standard deviation (RSD) for the confirmatory mass transition (m/z 406/188).
Calibration	Calibration range: 0.075 to 10 ng/mL $R^2 > 0.99$ The linear regression curve for difenoconazole standards in acetonitrile/water is y=29183.22 + 183584.28 x. The calibration curve ranges from 0.075 ng/mL up to 10 ng/mL standard concentration. The lower margin of the linearity was 30% of the LOQ, and the upper margin was at least 20% above the highest concentration in the final extracts.
Assessment of matrix effects	The response of the analyte obtained from the matrix-matched standard solutions was compared against the response obtained from solvent-based standard solutions to allow calculation of any matrix effect (either suppression or enhancement of response). Matrix effects for Difenoconazole were < ± 20% and therefore considered to be insignificant. Therefore solvent-based standard solutions were used for the quantification of Difenoconazole in honey.
Limit of determination/quantification	The limit of detection was estimated as 3 x baseline noise for both mass transitions. The LOD was approx. 0.0002 mg/kg (2% of the LOQ) for the quantification and confirmation mass transitions.

Conclusion
The data presented demonstrates that the analytical method GRM066.03A for the determination of residues of Difenoconazole is suitable for the determination of residues in honey with satisfactory accuracy, precision and repeatability using LC-MS/MS detection.
The method was successfully validated according to SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1.

Comments of zRMS:	The employed in the study analytical method for difenoconazole is accepted.

Reference:	KCP 5.1-06 (submitted as KCP 10.2.1-01)
Report	Effects of GLOB1911F on *Pseudokirchneriella subcapitata* in an algal growth inhibition test, Juckeland D., 2020a, report No 20 48 AAL 0002.
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Executive summary
Błąd! Nie można odnaleźć źródła odwołania. An in-house developed reversed phase HPLC-method with mass-spectrometric (MS/MS) detection was used for analysis of the test solutions from the biological part.

The method was validated according to SANCO/3029/99 rev. 4

HPLC conditions:

Instrument	A Shimadzu HPLC system with a triple quadrupole mass spectrometric detector was used
Mobile phase	A: water containing 1 mL/L formic acid B: methanol containing 1 mL/L formic acid
Flow rate	0.4 mL/min
Gradient	0.00 min 70% B 3.00 min 100% B 6.00 min 100% B 6.01 min 70% B
Run time	8 min
Injection Volume	1 µL
Column	ACE Excel3 C18-AR, 3 µm, 100 * 2.1 mm
Detection	MS: ESI positive, MRM, m/z = 405.9→250.95, 405.9→187.9, 405.9→337.0
Retention Time:	Approx. 3.4 min

Results and discussion
The method was validated with test medium / methanol (50:50, resembling stabilised samples), spiked with test item at 0.051 and 3.38 mg/L difenoconazole, corresponding to 0.102 and 7.76 mg/L in undiluted test solutions.

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

Validation level	Nominal concentration [mg/L]	Dilution factor	Analysed concentration [mg/L]	Mean analysed concentration [mg/L]	Mean recovery [%]	RSD [%]
Blank	0.0000	10	<30% LOQ* <30% LOQ*	<30% LOQ	---	---
Low	0.0508	10	0.0471 0.0499 0.0517 0.0510 0.0504	0.0500	99	3.5
High	3.384	40	3.390 3.403 3.392 3.339 3.384	3.381	100	0.7

* Validation blank samples had peak areas of less than 30 % of the lowest validated concentration.

Table A 3: Characteristics for the analytical method used for validation of Difenoconazole residues in OECD medium

	Difenoconazole
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.

	Difenoconazole
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 60% of the lowest to 120% of the highest validation concentration. N = 8
Calibration range	Calibration range: 3.05 to 101.8 µg/L R^2 > 0.999 Linearity was demonstrated over the whole calibration range from 3.05 to 101.8 µg/L with 8 concentration levels (corresponding to 0.061 – 8.14 mg/L in the test solutions, dilution factor 20 – 80). This covers a range from approximately 60% of the LOQ to 120% of the highest nominal concentration level, with a coefficient of determination greater than 0.999. The regression equation is y = 9340.066x-1548.988 .
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/3029/99.
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. LOQ = 5 µg/L in diluted analytical samples, corresponding to 0.10 mg/L in undiluted test solutions.

Conclusion

The method given above is suitable for the determination of Difenoconazole in test medium as the following criteria are fulfilled:

- Blank values did not exceed 30 % of the lowest validated concentration;
- Accuracy was tested by spiking test medium with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%;
- Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level;
- Linearity was tested for at least 5 points at a concentration range of at least ± 20 % of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Comments of zRMS:	The employed in the study analytical method for difenoconazole is accepted

Reference:	KCP 5.1-07 (submitted as KCP 10.6.2/01)
Report	Effect of GLOB1911F on seedling emergence and seedling growth of six non-target terrestrial plant species under greenhouse conditions, Kästner K. 2020a, Report No. 20 46 PSE 0002.
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Executive summary

Błąd! Nie można odnaleźć źródła odwołania. An in-house developed HPLC-method with DAD-detection was used for analysis of the test solutions from the biological part. The method was validated according to SANCO/3029/99 rev. 4

HPLC conditions:

Instrument	A Shimadzu LC-20 HPLC system equipped with a diode-array detector was used:
Mobile phase	A: Water with 0.1% (v/v) phosphoric acid B: Acetonitrile with 0.1% (v/v) phosphoric acid
Flow rate	0.50 mL/min
Gradient	0.00 min 50 % B 4.00 min 95 % B 6.00 min 95 % B 6.01 min 50 % B 8.00 min Stop
Injection Volume	5 µL
Column	ACE C18, 5µm, 150*2.1 mm
Oven temperature	40°C
Detection	UV-detection at 201 nm
Retention Time:	3.3 min for **Błąd! Nie można odnaleźć źródła odwołania.**

Results and discussion
The method was validated with test matrix spiked with test item at 52% of the nominal test concentration (1344 mg/L of difenoconazole) and at 120% of the nominal test concentration (3140 mg/L of difenoconazole).

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

Validation	Number of replicates	Nominal conc. of a.i. [mg/L]	Nominal conc. of a.i. regarding DF [mg/L]	Mean measured conc. of a.i. [mg/L]	Recalibration-factor (RCF)	Dilution factor (DF)	Mean analysed conc. of a.i. [mg/L]	Mean recovery [% of nominal]	RSD [%]
Validation low conc.	5	1344	13.44	12.98	1.002	100	1300	97	0.7
Validation high conc.	5	3140	31.40	30.91	1.003	100	3101	99	0.7

Table A 5: Characteristics for the analytical method used for validation of Difenoconazole residues in OECD medium

	Difenoconazole
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 19% of the lowest to 159% of the highest validation concentration. N = 6
Calibration range	Calibration range: 2.495 to 49.90 mg/L $R^2 > 0.99$ Linearity was tested for at least 5 points at a concentration range of at least +/-20% of a.i. in the analytical solution. The calibration function for difenoconazole was linear in the range of 2.495 to 49.90 mg/L. regression equation was: y = 67479.1x-13971.4 .

	Difenoconazole
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/3029/99.
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. LOQ = 1344 mg/L (13.44 mg/L regarding DF)

Conclusion

The method given above is suitable for the determination of Difenoconazole in test medium as the following criteria are fulfilled:

- Blank values did not exceed 30 % of the lowest validated concentration;
- Accuracy was tested by spiking test medium with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%;
- Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level;
- Linearity was tested for at least 5 points at a concentration range of at least ± 20 % of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Comments of zRMS:	The employed in the study analytical method for difenoconazole is accepted

Reference:	KCP 5.1-08 (submitted as KCP 10.6.2/02)
Report	Effect of GLOB1911F on vegetative vigour of six non-target terrestrial plant species under greenhouse conditions, Kästner K, 2020b, 20 46 PVV 0002.
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Executive summary

Błąd! Nie można odnaleźć źródła odwołania. An in-house developed HPLC-method with DAD-detection was used for analysis of the test solutions from the biological part. The method was validated according to SANCO/3029/99 rev. 4

HPLC conditions:

Instrument	A Shimadzu LC-20 HPLC system equipped with a diode-array detector was used:
Mobile phase	A: Water with 0.1% (v/v) phosphoric acid B: Acetonitrile with 0.1% (v/v) phosphoric acid
Flow rate	0.50 mL/min
Gradient	0.00 min 50 % B 4.00 min 95 % B 6.00 min 95 % B 6.01 min 50 % B 8.00 min Stop
Injection Volume	5 µL
Column	ACE C18, 5µm, 150*2.1 mm
Oven temperature	40°C
Detection	UV-detection at 201 nm
Retention Time:	3.3 min for **Błąd! Nie można odnaleźć źródła odwołania.**

Results and discussion

The method was validated with test matrix spiked with test item at 52% of the nominal test concentration (1344 mg/L of difenoconazole) and at 120% of the nominal test concentration (3140 mg/L of difenoconazole).

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

Validation	Number of replicates	Nominal conc. of a.i. [mg/L]	Nominal conc. of a.i. regarding DF [mg/L]	Mean measured conc. of a.i. [mg/L]	Recalibration-factor (RCF)	Dilution factor (DF)	Mean analysed conc. of a.i. [mg/L]	Mean recovery [% of nominal]	RSD [%]
Validation low conc.	5	1344	13.44	12.98	1.002	100	1300	97	0.7
Validation high conc.	5	3140	31.40	30.91	1.003	100	3101	99	0.7

Table A 6: Characteristics for the analytical method used for validation of Difenoconazole residues in OECD medium

	Difenoconazole
Specificity	The specificity of the method was assured by highly specific MS/MS-detection and the absence of interfering peaks. Validation blank samples had peak areas of less than 30% of the lowest validated concentration.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 19% of the lowest to 159% of the highest validation concentration. N = 6
Calibration range	Calibration range: 2.495 to 49.90 mg/L $R^2 > 0.99$ An external calibration with the analytical reference item was performed from 19% of the lowest validaiton measuring concentration of 159% of the highest validation measuring concentration for difenoconazole, (2.495 to 49.90 mg/L of difenoconazole). Matrix effects were not taken into account since the samples of the biological part as well as the validation samples were diluted with a dilution factor of 100. The regression equation is: y = 67479.1x -13971.4 .

	Difenoconazole
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/3029/99.
Limit of determination/quantification	Limit of quantification representing the lowest successfully validated fortification level. LOQ = 1344 mg/L (13.44 mg/L regarding DF)

Conclusion

The method given above is suitable for the determination of Difenoconazole in test medium as the following criteria are fulfilled:

- Blank values did not exceed 30 % of the lowest validated concentration;
- Accuracy was tested by spiking test medium with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%;
- Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level;
- Linearity was tested for at least 5 points at a concentration range of at least ± 20 % of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Comments of zRMS:	The employed in the study analytical method for difenoconazole is accepted

Reference:	KCP 5.1-09
Report	Acute toxicity of GLOB1911F to the bumblebee *Bombus terrestris L.* under laboratory conditions. Report number 20 48 BBA 0010
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Objective

The purpose of the analytical phase of the study was the verification of the concentration of difenoconazole in test item solutions of an acute toxicity test on bumblebees.

Materials and methods

An in-house developed method using reversed high performance liquid chromatography (RP-HPLC- coupled to a tandem mass spectrometer (LC-MS/MS). The method was validated according to SANCO/3029/99 rev. 4

HPLC conditions:

Instrument	An Agilent 1200 HPLC system with a 6460 triple quadruple mass spectrometric detector was used for determination.:
Mobile phase	A: Water with 0.1% formic acid and 5mM ammonium formate B: Methanol containing 0.1% formic acid
Flow rate	0.400 mL/min
Gradient	0.00 min 60 % B 5.00 min 100 % B 6.00 min Stop Posttime 3 min
Injection Volume	10 µL
Column	ACE Excel 3 C18, 2.1 x 100 mm, 3 µm
Detection	ESI positive, MRM m/z 406 → 337 m/z 406 → 251
Retention Time:	4.5 min

Results and discussion

The method was validated for both toxicity tests with the respective sample matrix fortified with the test item.

Table A 7: Summary of the validation results – contact toxicity test

Sample description	*Number of replicates*	*Nominal conc. of difenoconazole [mg/L]*	*Mean analysed conc. of difenoconazole [mg/L]*	*Mean recovery [% of nominal]*	*RSD [%]*
20CRB0031-Val-high-con	5	135897	144682	106	2.26
20CRB0031-Val-low-con	5	3126	2681	85.8	1.71
20CRB0031-Val-blank-con	2	0.000	< 30% LOQ	-	-

LOQ = 3126 mg/L of difenoconazole

Table A 8: Summary of the validation results – oral toxicity test

Sample description	*Number of replicates*	*Nominal conc. of difenoconazole [mg/L]*	*Mean analysed conc. of difenoconazole [mg/L]*	*Mean recovery [% of nominal]*	*RSD [%]*
20CRB0031-Val-high-oral	5	6870	6297	91.7	3.77
20CRB0031-Val-low-oral	5	151	132	87.5	2.76
20CRB0031-Val-blank-oral	2	0.000	< 30% LOQ	-	-

LOQ = 151 mg/L of difenoconazole

Table A 9: Characteristics for the analytical method used for validation of Difenoconazole residues in OECD medium

	Difenoconazole
Specificity	The specificity of the method was assured by multiple reaction monitoring (MRM) detection with two transitions and the absence of interfering peaks. The ratio of quantifier and qualifier ions was recorded and was constant withtin ± 20%. Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
Calibration (type, number of data points)	An external calibration with the analytical reference item was performed from 19% of the lowest to 159% of the highest validation concentration. N = 6

	Difenoconazole
Calibration range	Calibration range: 6.99 to 140 µg/L $R^2 > 0.99$ The calibration functions for difenoconazole were quadratic in the range of 6.99 to 140 µg/L. For the contact toxicity test the regression equation is: $y = -0.659 x^2 + 3438.3x + 5950.7$, for the oral toxicity test the regression equatin is: $y = -1.8 x^2 + 3036.44 x + 811.28$.
Assessment of matrix effects is presented	Yes The recovery and precision data show that the influences of test medium were within the limits of the guidance document SANCO/3029/99.
Limit of determination/quantification	Limit of quantification (LOQ) representing the lowest successfully validated fortification level. LOQ = 3126 mg/L difenoconazole in sample matrix water containing 0.5% triton-X LOQ = 151 mg/L difenoconazole in sample matrix sucrose solution containing 50% sucrose.

Conclusion

The method given above is suitable for the determination of Difenoconazole in test medium as the following criteria are fulfilled:

- Blank values did not exceed 30 % of the lowest validated concentration;
- Accuracy was tested by spiking test medium with test item at 2 concentrations levels. Mean recoveries for each level were in the range 70-110%;
- Repeatability with 5 replicates for each level with the RSD (relative standard deviation) was < 20% per level;
- Linearity was tested for at least 5 points at a concentration range of at least ± 20 % of a.i. in the analytical solution, with correlation coefficient of > 0.99.

Comments of zRMS:	No original report of the study. Based on the description of the study the analytical part of the study is not accepted.

Reference:	KCP 5.1-10
Report	Difenoconazole - Effects on the Development of Sediment-Dwelling Larvae of Chironomus riparius in Water-Sediment Systems with Spiked Sediment Report Number D81747
Guideline(s):	OECD Guidelines for the Testing of Chemicals: Test Guideline 218: Sediment-Water Chironomid Toxicity Test using Spiked Sediment (2004)
GLP:	Yes
Acceptability:	No

Executive summary

The effects of difenoconazole on the development of *Chironomus riparius* were determined under static conditions. Organisms were exposed to nominal concentrations of 5.0, 10, 20, 40 and 80 mg difenoconazole/kg dry sediment (initial measured concentrations 3.7, 8.2, 14, 28 and 66 mg difenoconazole/kg dry sediment, respectively) alongside a dilution water control and a solvent control.
Based on initial measured concentrations, the 28 day EC50 for emergence ratio was 36 mg difenoconazole/kg dry sediment and the 28 day EC50 for male, female and pooled sexes development rate was >66 mg difenoconazole/kg dry sediment. The 28 day NOEC for emergence ratio was 14 mg difenoconazole/kg dry sediment, and for male, female and pooled sexes development rate were 8.2, 28 mg and 8.2 mg

difenoconazole/kg dry sediment, respectively. Thus, the overall 28-day NOEC was 8.2 mg difenoconazole/kg dry sediment. The overall 28 day LOEC was 14 mg difenoconazole/kg dry sediment, due to a reduced development rate of the male midges.

Materials and methods

Test Material	
Description:	Difenoconazole tech CGA169374 tech
Lot/Batch #:	SMO3A0011
Purity:	96.6% w/w
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/Expiry date:	30 June 2015
Treatments	
Test concentrations:	Dilution water control, solvent control and nominal concentrations of 5.0, 10, 20, 40 and 80 mg difenoconazole/kg dry sediment (3.7, 8.2, 14, 28 and 66 mg difenoconazole/kg dry sediment, initial measured concentrations, respectively)
Solvent:	Acetone
Analysis of test concentrations:	Yes (0, 7 and 28 days) – based on measurement of difenoconazole using HPLC-MS/MS
Test organism	
Species:	*Chironomus riparius*, first instar (2-3 days old)
Source:	Continuous laboratory cultures, original source not reported
Feeding:	Fish food (Tetra Min®) suspension in test water at least three times per week until day 27, when all adult midges had emerged.
Test design	
Test vessels:	Glass vessels (600 mL, approximately 8 cm diameter) covered with lid containing mosquito net and containing 89 g of dry sediment and 250 mL of test medium
Test medium:	Reconstituted water (“M7 – medium”)
Artificial Sediment:	5% sphagnum peat (air dried and finely ground to ≤1 mm) 20% kaolin clay (content of Al_2O_3 35.5%) 75% Sihelco 36 sand (>99% of the particles between 90 and 250 µm) 0.35% Calcium carbonate The total organic carbon content of the final sediment mixture was 2.4%

Results and discussion

At all test concentrations, the difenoconazole concentrations in the test media one hour after application ranged between 71 to 82% of the nominal values, and ranged between 70 to 85% at test termination (see table below). The Limit of Quantification (LOQ) for sediment and water analysis was 0.452 µg difenoconazole/kg dry sediment. Biological results are based on the initial measured difenoconazole concentrations, calculated as concentrations of the test item in the dry sediment.

Table A3: Analytical results

Day	Nominal concentration (mg difenoconazole/kg dry sediment)	Measured concentration (mg difenoconazole/kg dry sediment)	% of nominal
0 (fresh)	Solvent control	n.a.	n.a.
	5	3.66	73
	10	8.19	82
	20	14.4	72
	40	28.4	71
	80	65.7	82
7 (old)	Solvent control	n.a.	n.a.
	5	4.31	86
	10	8.97	90
	20	14.8	74
	40	32.6	82
	80	77.3	97
28 (old)	Solvent control	n.a.	n.a.
	5	4.26	85
	10	7.70	77
	20	14.4	72
	40	31.0	77
	80	56.0	70

n.a. not applicable
The tabulated values of the samples represent results obtained by calculation using the exact raw data

Conclusion

Based on initial measured concentrations, the 28 day EC_{50} for emergence ratio was 36 mg difenoconazole/kg dry sediment and the 28 day EC_{50} for male, female and pooled sexes development rate was >66 mg difenoconazole/kg dry sediment. Monograph (DRAR) Volume III Chapter 9 **B9 (AS)** 97 **Difenoconazole** August 2018
The 28 day NOEC for emergence ratio was 14 mg difenoconazole/kg dry sediment, and for male, female and pooled sexes development rate were 8.2, 28 mg and 8.2 mg difenoconazole/kg dry sediment, respectively, hence the overall 28-day NOEC was 8.2 mg difenoconazole/kg dry sediment.
The overall 28 day LOEC was 14 mg difenoconazole/kg dry sediment due to a reduced development rate of the male midges.

Comments of zRMS:	No original report of the study. Based on the description of the study the study analytics is accepted.

Reference:	KCP 5.1-11
Report	Chronic toxicity of GLOB1911F to the honey bee *Apis mellifera L.* under laboratory conditions Verification of the concentration of difenoconazole in feeding solutions by LC-MS/MS Report number 20 48 BAC 0003
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
GLP:	Yes
Acceptability:	Yes

Summary

The purpose of the analytical phase of the study was the verification of the concentration of difenoconazole in feeding solutions (sucrose solution containing 50% (w/v) sucrose + 0.1% (w/v) xanthan) of a honey bee

chronic oral toxicity study. The determination was conducted by an in-house developed method using reversed phase high performance liquid chromatography (RP-HPLC) coupled to a tandem mass spectrometer (LC-MS/MS).
The analytical method was validated according to SANCO/3029/99 rev. 4.

Summary of method validation results:

Sample description	*Number of replicates*	*Nominal conc. of difenoconazole [mg/kg]*	*Mean analysed conc. of difenoconazole [mg/kg]*	*Mean recovery [% of nominal]*	*RSD [%]*
20CRB0005-Val-high	5	2861	3074	107	3.20
20CRB0005-Val-low	5	97.3	96.2	98.9	1.80
20CRB0005-Val-blank	2	0.000	< 30% LOQ	-	-

LOQ = 97.3 mg/kg of difenoconazole

Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.
The specificity of the method was assured by multiple reaction monitoring (MRM)-detection with two transitions and the absence of interfering peaks. The ratio of quantifier and qualifier ions was recorded and was constant within ± 20%.
The recovery and precision data show that the influences of sample matrix were within the limits of the guidance document SANCO/3029/99 rev.4; all criteria were fulfilled:
– blank values did not exceed 30% of the lowest validated concentration,
– mean recoveries for each level were in the range 70-110%,
– the RSD was < 20% per level.
The limit of quantification (LOQ) was defined in the context of this analytical phase as the lowest successfully validated fortification level (97.3 mg/kg of difenoconazole, corresponding to 38.3 µg/L regarding the applied dilution factor for the validation low samples).

Analysis results

Sample identification	*Nominal conc. of difenoconazole [mg/kg]*	*Analysed conc. of difenoconazole [mg/kg]*	*Recovery [% of nominal]*
20BAC0003-D0-BC-A	0.000	< 30% LOQ	-
20BAC0003-D0-AT-A	2125	2124	100
20BAC0003-D0-ET-A	203	209	103

LOQ = 97.3 mg/kg of difenoconazole

The recoveries of difenoconazole in the samples were 100% and 103%. No difenoconazole was detected in the control sample. Thus, the concentrations of difenoconazole in the feeding solutions from the biological part were verified.

Comments of zRMS:	The study analytics is accepted.

Reference:	KCP 5.1-12
Report	GLOB1911F – Repeated exposure of honey bee (Apis mellifera L.) larvae under laboratory conditions Verification of the concentration of difenoconazole in test item stock solutions by LC-MS/MS Report number 20 48 BLC 0004
Guideline(s):	SANCO/3029/99 rev. 4 (11/07/2000)
GLP:	Yes
Acceptability:	Yes

Summary

The purpose of the analytical phase of the study was the verification of the concentration of difenoconazole in test item stock solutions of a honey bee larvae chronic toxicity test. The determination was conducted by an in-house developed method using reversed phase high performance liquid chromatography (RP-HPLC) coupled to a tandem mass spectrometer (LC-MS/MS).

The specimens were present in two different aqueous sugar solutions (ASS):

ASS for diet B: 15% (w/v) glucose, 15% (w/v) fructose and 3% (w/v) yeast extract

ASS for diet C: 18% (w/v) glucose, 18% (w/v) fructose and 4% (w/v) yeast extract

The analytical method was validated according to SANCO/3029/99 rev. 4.

Summary of method validation results:

Sample description	*Number of replicates*	*Nominal conc. of difenoconazole [mg/kg]*	*Mean analysed conc. of difenoconazole [mg/kg]*	*Mean recovery [% of nominal]*	*RSD [%]*
20CRB0006-Val-high	5	2148	2036	94.8	2.47
20CRB0006-Val-low	5	19.3	18.2	94.2	1.85
20CRB0006-Val-blank	2	0.000	< 30% LOQ	-	-

LOQ = 19.3 mg/kg of difenoconazole

Validation blank samples had peak areas of less than 30% of the lowest validated concentration. No interfering peaks were detected.

The specificity of the method was assured by multiple reaction monitoring (MRM)-detection with two transitions and the absence of interfering peaks. The ratio of quantifier and qualifier ions was recorded and was constant within ± 20%.

The recovery and precision data show that the influences of sample matrix were within the limits of the guidance document SANCO/3029/99 rev.4; all criteria were fulfilled:

– blank values did not exceed 30% of the lowest validated concentration,
– mean recoveries for each level were in the range 70-110%,
– the RSD was < 20% per level.

The limit of quantification (LOQ) was defined in the context of this analytical phase as the lowest successfully validated fortification level (19.3 mg/kg of difenoconazole, corresponding to 19.0 µg/L regarding the applied dilution factor for the validation low samples).

Analysis results

Sample identification	*Nominal conc. of difenoconazole [mg/kg]*	*Analysed conc. of difenoconazole [mg/kg]*	*Recovery [% of nominal]*
20BLC0004-D3-C-A	-	-	-
20BLC0004-D4-C-A	-	-	-
20BLC0004-D5-C-A	-	-	-
20BLC0004-D6-C-A	-	-	-
20BLC0004-D3-StA-A	1589	1555	97.9
20BLC0004-D4-StA-A	1589	1710	108
20BLC0004-D5-StA-A	1589	1607	101
20BLC0004-D6-StA-A	1589	1562	98.3
20BLC0004-D3-StE-A	40.7	37.6	92.5
20BLC0004-D4-StE-A	40.7	45.0	111
20BLC0004-D5-StE-A	40.7	36.7	90.1
20BLC0004-D6-StE-A	40.7	39.4	96.9

LOQ = 19.3 mg/kg of difenoconazole

The recoveries of difenoconazole in the samples were between 90.1% and 111%. No difenoconazole was detected in the control samples. Thus, the concentrations of difenoconazole in the test item stock solutions

from the biological part were verified.

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

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

A 2.1.2.1.1.1 Independent laboratory validation

This study (Austin R., 2009a) has already been submitted in Poland (zRMS) to support the authorisation of the registered products Difcor 250 EC and Narita and was positively evaluated. For completeness, the summary is given below once again.

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 18.01.2014 prepared by Eko-Futura Sp. z o.o; IOR for the Step 2 of Difcor 250 EC. The following conclusion was made (cited by translation): "*The obtained results for individual components meet the requirements guidelines SANCO / 825/00 rev.7 and SANCO / 3029/99 rev.4 11 July 2000. The proposed method can be used by independent laboratories and may lead to the same results as the xxxxxx method, 2008.*"

Reference:	KCP 5.2-01
Report	Independent Laboratory Validation of a Method for the determination of difenoconazole in crops, Austin R., 2009a, CEMS-4180
Guideline(s):	Yes (SANCO/825/00 rev. 7, SANCO/3029/99 rev. 4, EPA OPPTS 860.1340 Residue Analytical Method. Test Guideline August 1996)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

This study consists in an ILV of the method presented in the validation report R A8143 for the determination of Difenoconazole in crops. The validation was performed for apples/fruits and oilseed rape/seeds because the extraction procedure in the validation study was identical for the tested crop matrices. Adjustments to the methodology were made:

- a HPLC system was used instead of a UPLC system and an equivalent HPLC column was used;
- the mobile phase gradient was modified due to the greater retention time of the analyte in HPLC

<u>HPLC-MS/MS conditions:</u>

7.1 Instrumentation
Agilent 1100 Series Liquid Chromatography System
API MS/MS System, Applied Biosystems

7.2 Operating Conditions

Mobile Phase A:	0.1% formic acid in milli Q water
Mobile Phase B:	0.1% formic acid in methanol
HPLC Column:	Waters XBridge 50 mm × 2.1 mm C18 5 µm Part number: 186003108
Column Oven Temp:	40°C
Flow Rate:	400 µL/min
Injection Volume:	10 µL

Gradient:

Total Time (min)	A %	B %
0.00	90.0	10.0
6.00	0.0	100.0
7.50	0.0	100.0
7.90	90.0	10.0
10.00	90.0	10.0

MS Parameters

Scan Type:	MRM
Ion Source:	Turbo Spray
Polarity:	Positive
CAD:	8.00
CUR:	24.00
Gas 1:	20.00
Gas 2:	30.00
IS:	5500.00
Temperature:	425°C
DP:	60.00
EP:	10.00
CXP:	8.40
Dwell (msec):	50.00

Compound Specific MS Parameters

Active Ingredient	Quantitation			Confirmatory		
	Q1	Q3	CE	Q1	Q3	CE
Difenoconazole	406.20	251.20	35.00	406.20	337.20	25.00

Results and discussions

Validation data:	
Specificity/ interferences :	Chromatograms of standards solution, of matrix fortified, of control specimen of apple and oil seed rape are given in the dossier. No interferences are observed at the retention time of difenoconazole. The method is considered as specific. A highly specific detection system was used (MS/MS) with 1 quantifier and 1 qualifier ion. Therefore, the method is highly specific for **Błąd! Nie można odnaleźć źródła odwołania.**.
Linearity :	The detector response for **Błąd! Nie można odnaleźć źródła odwołania.** analysis w as linear within the range from 2.5 ng/mL to 60 ng/mL (n=7) with $r^2 > 0.999$ (y = 4014 + 14875660 x for m/z 406 → 251 and y = 460 + 1860562 x for m/z 406 → 337).
Accuracy / repeatability :	The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation:

Difenoconazole in Apple (Quantitation *m/z* 406 → 251)

Fortification Level (mg/kg)	Recoveries			Number of Samples	Overall Recovery	
	Single Values (%)	Mean (%)	RSD (%)		Mean (%)	RSD (%)
0.01	106, 106, 105, 109, 99	105	3.6	5	97	10.2
0.1	86, 89, 82, 87, 96	88	5.7	5		

Difenoconazole in Apple (Confirmatory *m/z* 406 → 337)

Fortification Level (mg/kg)	Recoveries			Number of Samples	Overall Recovery	
	Single Values (%)	Mean (%)	RSD (%)		Mean (%)	RSD (%)
0.01	110, 102, 104, 105, 102	105	3.2	5	94	12.5
0.1	79, 84, 81, 82, 92	84	5.8	5		

Difenoconazole in Oil Seed Rape (Seeds) (Quantitation *m/z* 406 → 251)

Fortification Level (mg/kg)	Recoveries			Number of Samples	Overall Recovery	
	Single Values (%)	Mean (%)	RSD (%)		Mean (%)	RSD (%)
0.01	71, 70, 71, 73, 68	70	3.8	5	70	3.6
0.1	71, 73, 69, 66, 71	70	3.8	5		

Difenoconazole in Oil Seed Rape (Seeds) (Confirmatory *m/z* 406 → 337)

Fortification Level (mg/kg)	Recoveries			Number of Samples	Overall Recovery	
	Single Values (%)	Mean (%)	RSD (%)		Mean (%)	RSD (%)
0.01	75, 65, 71, 78, 68	71	7.1	5	70	5.3
0.1	70, 71, 69, 67, 71	70	2.8	5		

Conclusion

The ILV method was successfully performed which means that the method is suitable for determination of difenoconazole in apple/fruit and oilseed rape/seeds (as well as in carrots/roots and grapes/berry).

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

A 2.1.2.2.1 Analytical method 1 – LC/MS-MS

A 2.1.2.2.1.1 Method validation

Reference is made to study KCP 5.1-03 (A2.1.1.1.3.1).

A 2.1.2.2.1.2 Independent laboratory validation

This study (xxxxxx., 2009b) have already been submitted in Poland to support the authorisation of the registered products Difcor 250 EC and Narita and was positively evaluated. For completeness, the summary is given below once again.

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The study has been positively evaluated in Part B Section 2 of the Registration Report from 18.01.2014 prepared by Eko-Futura Sp. z o.o; IOR for the Step 2 of Difcor 250 EC. The following conclusion was made (cited by translation): *"The obtained results for individual components meet the requirements guidelines SANCO / 825/00 rev.7 and SANCO / 3029/99 rev.4 11 July 2000. The proposed method can be used by independent laboratories and may lead to the same results as the xxxxxx method, 2008."*

Reference:	KCP 5.1-02
Report	Independent Laboratory Validation of a Method for the determination of difenoconazole and its metabolite CGA205375 in bovine liver, kidney, muscle and fat, milk and eggs, xxxxxxx., 2009b, CEMS-4181.
Guideline(s):	Yes (SANCO/825/00 rev. 7, SANCO/3029/99 rev. 4, EPA OPPTS 860.1340 Residue Analytical Method. Test Guideline August 1996)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

This study consists in an ILV of the method presented in the validation report R A8143 for the determination of Difenoconazole and CGA205375 in bovine liver, kidney, muscle and fat, milk and eggs. Adjustments to the methodology were made:
- a HPLC system was used instead of a UPLC system and an equivalent HPLC column was used;
- the mobile phase gradient was modified due to the greater retention time of the analyte in HPLC

<u>HPLC-MS/MS conditions:</u>

7.1 Instrumentation
Agilent 1100 Series Liquid Chromatography System
API MS/MS System, Applied Biosystems

7.2 Operating Conditions

Mobile Phase A:	0.1% formic acid in milli Q water
Mobile Phase B:	0.1% formic acid in methanol
HPLC Column:	Waters XBridge 50 mm × 2.1 mm C18 5 µm Part number: 186003108
Column Oven Temp:	40°C
Flow Rate:	400 µL/min
Injection Volume:	10 µL

Gradient:

Total Time (min)	A %	B %
0.00	90.0	10.0
6.00	0.0	100.0
7.50	0.0	100.0
7.90	90.0	10.0
10.00	90.0	10.0

MS Parameters

Scan Type:	MRM
Ion Source:	Turbo Spray
Polarity:	Positive
CAD:	8.00
CUR:	24.00
Gas 1:	20.00
Gas 2:	30.00
IS:	5500.00
Temperature:	425°C
DP:	60.00
EP:	10.00
CXP:	8.40
Dwell (msec):	50.00

Compound Specific MS Parameters

Active Ingredient	Quantitation			1st Confirmatory			2nd Confirmatory		
	Q1	Q3	CE	Q1	Q3	CE	Q1	Q3	CE
Difenoconazole	406.20	251.20	35.00	406.20	337.20	25.00	N/A		
CGA205375	352.00	283.00	25.00	352.10	70.00	54.00	350.10	70.00	54.00

Results and discussions

Validation data:	
Specificity/ interferences :	Chromatograms of standards solution, of matrix fortified and of control specimen of animal matrice (bovine liver, bovine kidney, bovine muscle, fat, eggs and milk) are given in the dossier for Difenoconazole and CGA205375. No interferences are observed at the retention time of difenoconazole and CGA205375. The method is considered as specific. A highly specific detection system was used (MS/MS) with 1 quantifier and 1 qualifier ion. Therefore, the method is highly specific for **Błąd! Nie można odnaleźć źródła odwołania.** and CGA205375.
Linearity :	The detector response for **Błąd! Nie można odnaleźć źródła odwołania.** and CGA205375 analysis was linear within the range from 2.5 ng/mL to 60 ng/mL (n>5) with $r^2 > 0.999$ (Difenoconazole: y = 9186 + 20136965 x for m/z 406 → 251 and y = 66 + 2306819 x for m/z 406 → 337 // CGA205375: y = 1827 + 10566919 x for m/z 350 → 70, y = 959 + 6840708 x for m/z 352 → 70 and y = 55 + 184602 x for m/z 352 → 283).

Accuracy / repeatability :

The method is accurate and repeatable with the following values of percentage recovery +/- relative standard deviation:

Difenoconazole Quantitation

Specimen Type	Number of Samples	Fortification Level (mg/kg)	Recoveries		Overall Recovery for Each Specimen Type	
			Mean (%)	RSD (%)	Mean (%)	RSD (%)
Bovine Liver	5	0.01	75	8.3	75	8.3
	5	0.1	75	9.3		
Bovine Kidney	5	0.01	92	3.3	100	9.7
	5	0.1	108	6.5		
Bovine Muscle	5	0.01	81	10.2	79	10.0
	5	0.1	77	10.3		
Bovine Fat	5	0.01	83	4.3	84	3.6
	5	0.1	85	3.0		
Milk	5	0.01	76	10.7	88	16.1
	5	0.1	99	5.3		
Eggs	5	0.01	86	6.5	78	11.9
	5	0.1	70	3.7		

Difenoconazole Confirmatory

Specimen Type	Number of Samples	Fortification Level (mg/kg)	Recoveries		Overall Recovery for Each Specimen Type	
			Mean (%)	RSD (%)	Mean (%)	RSD (%)
Bovine Liver	5	0.01	75	6.7	73	8.1
	5	0.1	71	9.2		
Bovine Kidney	5	0.01	86	3.8	92	9.0
	5	0.1	98	7.5		
Bovine Muscle	5	0.01	82	11.2	79	11.0
	5	0.1	75	9.8		
Bovine Fat	5	0.01	81	5.0	82	4.2
	5	0.1	83	3.4		
Milk	5	0.01	76	9.5	86	14.1
	5	0.1	96	5.7		
Eggs	5	0.01	84	8.0	77	11.3
	5	0.1	70	2.6		

CGA205375 Quantitation

Specimen Type	Number of Samples	Fortification Level (mg/kg)	Recoveries		Overall Recovery for Each Specimen Type	
			Mean (%)	RSD (%)	Mean (%)	RSD (%)
Bovine Liver	5	0.01	82	8.2	78	10.3
	5	0.1	74	10.4		
Bovine Kidney	5	0.01	84	4.8	89	8.3
	5	0.1	95	5.9		
Bovine Muscle	5	0.01	77	9.5	76	9.2
	5	0.1	74	9.4		
Bovine Fat	5	0.01	86	6.2	89	5.5
	5	0.1	92	2.4		
Milk	5	0.01	87	6.4	89	5.3
	5	0.1	91	3.3		
Eggs	5	0.01	90	11.1	92	7.9
	5	0.1	94	2.9		

CGA205375 1st Confirmatory

Specimen Type	Number of Samples	Fortification Level (mg/kg)	Recoveries		Overall Recovery for Each Specimen Type	
			Mean (%)	RSD (%)	Mean (%)	RSD (%)
Bovine Liver	5	0.01	80	7.1	77	9.7
	5	0.1	73	10.9		
Bovine Kidney	5	0.01	85	5.7	89	7.1
	5	0.1	93	5.5		
Bovine Muscle	5	0.01	75	11.1	75	9.8
	5	0.1	74	9.4		
Bovine Fat	5	0.01	87	6.3	89	5.4
	5	0.1	91	3.4		
Milk	5	0.01	86	6.7	88	5.7
	5	0.1	91	2.9		
Eggs	5	0.01	90	11.9	91	8.0
	5	0.1	92	2.4		

CGA205375 2nd Confirmatory

Specimen Type	Number of Samples	Fortification Level (mg/kg)	Recoveries		Overall Recovery for Each Specimen Type	
			Mean (%)	RSD (%)	Mean (%)	RSD (%)
Bovine Liver	5	0.01	75	8.3	73	9.8
	5	0.1	71	11.6		
Bovine Kidney	5	0.01	87	7.8	89	6.7
	5	0.1	91	5.1		
Bovine Muscle	5	0.01	79	12.6	76	11.0
	5	0.1	74	8.9		
Bovine Fat	5	0.01	90	9.9	91	7.1
	5	0.1	92	3.8		
Milk	5	0.01	88	10.4	89	7.5
	5	0.1	90	3.9		
Eggs	5	0.01	87	12.8	89	9.1
	5	0.1	92	3.3		

LOQ	The LOQ of the method is 0.01mg/kg for the determination of Difenoconazole and CGA205375 in muscle, kidney, milk, eggs, fat, and liver.

Conclusion

The ILV method was successfully performed which means that the method is suitable for determination of

Difenoconazole and its metabolite CGA205375 in bovine liver, kidney, muscle and fat, milk and eggs.

A 2.1.2.2.1.3 Confirmatory method (if required)

This study (Faessel F., 2013a) have already been submitted in Poland (zRMS) to support the authorisation of the registered product Difcor 250 EC and was positively evaluated. For completeness, the summary is given below once again.

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 0.7.03.2016 prepared by Eko-Futura Sp. z.0.0; IOR for the extension of authorisation of product Difcor 250 EC for minor uses. The following conclusion was made: *"To be fit for the intended purpose, the method was successfully evaluated and met certain validation characteristics: specificity, linearity, repeatability and accuracy. The analytical method fulfils the criteria of SANCO/825/00 rev. 8.1.* *The study has been performed in compliance with Good Laboratory Practice and is acceptable."*

Reference:	KCP 5.2-03
Report	Validation of the Analytical Method for the Determination of Difenoconazole Residues in milk, Faessel V., 2013a, R B2295
Guideline(s):	Yes (SANCO/825/00 rev. 8.1, SANCO/3029/99 rev. 4)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The method is the same as in study (xxxxxx., 2008a) and has been validated with the same laboratory. Difenoconazole were extracted from samples using an Anadiag method:
For the preparation and extraction of the samples: SOP MP 290
For the analysis of extracts and for the calibration: SOP MA 616
The principle of the method is based on a manual or automatic extraction of the crop with acetone, followed by a clean-up of the crude extract by a liquid/liquid partition with dichloromethane. The residues are dissolved in hexane/ethanol and then quantified by LC-MS/MS

LC-MS/MS conditions:

Analytical Conditions
LC-MS/MS

n° MA_616-03

Apparatus	LC /MS /MS QPREMIER	
	APPARATUS	**MANUFACTURER**
UPLC system	Acquity Column Manager	WATERS
	Acquity Binary Sample Manager	WATERS
	Acquity Binary Solvent Manager	WATERS
	Acquity Binary Sample Organizer	WATERS
Detector	Quattro Premier	WATERS

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1*100 mm	Supplier reference	186002352	Temperature	40 °C
Development Column ANADIAG Number	166	Stationary Phase	C18	Comment	-

Mobile phase

A =	H_2O MilliQ + 0.1 % formic acid	C =	-
B =	Methanol + 0.1 % formic acid	D =	-

Sample temperature	15 ° C

Elution

Elution	*Time*	*Flow*	Composition (%)				*Curve*	Elution	*Time*	*Flow*	Composition (%)				*Curve*
	min	mL/min	A	B	C	D	(type)		min	mL/min	A	B	C	D	(type)
Pg1	0.00	0.4	90	10	-	-	0	Pg5	8.00	0.4	90	10	-	-	6
Pg2	6.00	0.4	0	100	-	-	6	Pg6	-	-	-	-	-	-	-
Pg3	6.50	0.4	0	100	-	-	6	Pg7	-	-	-	-	-	-	-
Pg4	6.90	0.4	90	10	-	-	6	Pg8	-	-	-	-	-	-	-

Detector

IONISATION mode*	ES x	APCI
Polarity*	Pos x	Neg

*make a cross in the right choice

Active ingredient(s)	Cone voltage	Collision Energy	Dwell time (ms)	TRANSITION 1 Parent > Daughter	TRANSITION 2 Parent > Daughter	RT (min.)
DIFENOCONAZOLE	40	20	50	405.5>250.5	-	5.63
	40	15	50	-	405.5>336.5	5.63

Date of application of analytical conditions

Study	B2295	Column ANADIAG number	166
Matrix	milk	Retention time	Difenoconazole: ≈ 5.63 min.
Sample temperature	+15 °C	Injected volume	10 µL

Results and discussions

Validation data:

Specificity

Chromatograms of control samples, of standard solution and of fortified samples are provided. No interference > 30% LOQ is observed at the retention time of difenoconazole. The specificity of the method is acceptable.

Linearity

For quantitation the detector response for difenoconazole analysis was linear within the range from 0.7 ng/mL to 31.4 ng/mL with $r^2 > 0.999$ (y = - 0.09 + 0.0012153 x).

For confirmation the detector response for difenoconazole analysis was linear within the range from 0.7 ng/mL to 31.4 ng/mL with $r^2 > 0.999$ (y = - 0.19 + 0.003431 x).

Accuracy

% RSD and % mean recoveries are reported below:

Quantitation:

Analyte	**Matrix**	**Fortification level (mg/kg)**	**Mean recovery Percentage (%)**	**Standard deviation (SD) (%)**	**Relative standard deviation (RSD) (%)**	**Number of fortified samples (n)**
Difenoconazole	Milk	0.005	82.5%	6.8%	8.3%	5

Confirmation:

Analyte	Matrix	Fortification level (mg/kg)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	milk	0.005	**91.2%**	**6.6%**	**7.3%**	5

LOQ
The LOQ of the method is 0.005mg/kg.

Conclusion

An analytical method (xxxxxx, 2008 and Faessel V 2013) has been provided for the determination of difenoconazole residue in milk with LOQ=0.005mg/L. The method is considered as highly specific.

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

This study (Eberhard Z., 2008) have already been submitted in Poland (zRMS) to support the authorisation of the registered product Difcor 250 EC and was positively evaluated. For completeness, the summary is given below once again.

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable. The validation study has been previously evaluated for the product Difcor 250 EC in France and submitted in Poland for the product Difure Pro: "*An analytical method (Eberhard Zietz – 2008) for the determination of Difenoconazole and CGA 205375 in soil has already been provided for the preparation DIFCOR 250 EC and validated by LC-MS/MS according to SANCO 825/00 rev 8.1 with* ***LOQ=0.001mg/kg*** *for each analyte. The method is considered as highly specific. No confirmatory method is required.*"

Reference:	KCP 5.2-04
Report	Validation of an Analytical Method for the Determination of Difenoconazole and its Metabolite CGA 205375 in Soil, Eberhard Z., 2008, IF-08/01135942
Guideline(s):	Yes (SANCO/825/00 rev. 8.1)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analyte difenoconazole was extracted with methanol/aqueous ammonium formiate solution (pH 8) (7:3, v:v) and determined by analysing the characteristic MS/MS transition m/z 406 →m/z 251 for quantification and MS/MS transition m/z 406 → m/z 337 for confirmation.
The metabolite CGA 205375 was extracted with acetonitrile/aqueous ammonium formiate solution (pH 8) (8:2, v:v) and determined by analysing the characteristic MS/MS transition m/z 350 →m/z 70 for quantification and MS/MS transition m/z 352 → m/z 70 for confirmation (ionisation mode: API MS/MS)

Results and discussions

Validation data

Specificity
Chromatograms of standards solution, of matrix fortified and of control samples are given in the dossier for both analytes. No interferences are observed at the retention time of difenoconazole and CGA205375. The method is considered as specific.

Linearity
The detector response for difenoconazole analysis was linear within the range from 0.05 ng/mL to 5 ng/mL (n=7) with $r^2 > 0.999$

Transition m/z 406 →m/z 251
Y=997.8+8693563.24*X r = 0.999
Transition m/z 406 → m/z 337
Y=224.9+2040742.41*X r = 0.999

The detector response for CGA205375 was linear within the range from 0.05 ng/mL to 5 ng/mL (n=7) with $r^2 > 0.999$

Transition m/z 350 → m/z 70 :
Y=774.5+1055270.54*X r = 0.999

Transition m/z 352 → m/z 70:
Y=464+636610.4*X r = 0.999

The linearity of the method is acceptable

Accuracy

For Difenoconazole:

Specimen No.	Fortified [mg/kg]	Primary Method Transition m/z 406 → m/z 251 Analysed [mg/kg]	Primary Method Transition m/z 406 → m/z 251 Recovery [%]	Confirmatory method Transition m/z 406 → m/z 337 Analysed [mg/kg]	Confirmatory method Transition m/z 406 → m/z 337 Recovery [%]
010/8234674-1	control	< 0.001	-	< 0.001	-
010/8234674-2	control	< 0.001	-	< 0.001	-
010/8234674-A	0.00107	0.00093	87	0.00093	87
010/8234674-B	0.00107	0.00091	86	0.00091	85
010/8234674-C	0.00106	0.00089	84	0.00088	83
010/8234674-D	0.00106	0.00090	84	0.00091	85
010/8234674-E	0.00106	0.00113	106	0.00114	107
010/8234674-F	0.01060	0.00895	84	0.00904	85
010/8234674-G	0.01062	0.00878	83	0.00903	85
010/8234674-H	0.01060	0.00739	70	0.00746	70
010/8234674-K	0.01069	0.00945	88	0.00963	90
010/8234674-L	0.01066	0.00944	89	0.00948	89
Fortification:		Average [%]:	89		89
0.001 mg/kg		Standard deviation [%]	9.6		10.1
		Relative standard deviation [%]:	10.7		11.3
		Number of determinations:	5		5
Fortification:		Average [%]:	83		84
0.01 mg/kg		Standard deviation [%]	7.7		7.9
		Relative standard deviation [%]:	9.3		9.4
		Number of determinations:	5		5

For CGA 205375:

Specimen No.	Fortified [mg/kg]	Primary Method Transition m/z 350 → m/z 70 Analysed [mg/kg]	Primary Method Transition m/z 350 → m/z 70 Recovery [%]	Confirmatory method Transition m/z 352 → m/z 70 Analysed [mg/kg]	Confirmatory method Transition m/z 352 → m/z 70 Recovery [%]
010/8234674-3	control	< 0.001	-	< 0.001	-
010/8234674-4	control	< 0.001	-	< 0.001	-
010/8234674-M	0.000992	0.001092	110	0.00098	99
010/8234674-N	0.000993	0.000914	92	0.00103	103
010/8234674-O	0.000995	0.000924	93	0.00090	91
010/8234674-P	0.000993	0.000831	84	0.00087	87
010/8234674-Q	0.000995	0.000496	(50)*	0.00053	(53)*
010/8234674-R	0.009970	0.00950	95	0.00945	95
010/8234674-S	0.009920	0.00930	94	0.00933	94
010/8234674-T	0.009911	0.00958	97	0.00925	93
010/8234674-U	0.009940	0.009436	95	0.00970	98
010/8234674-V	0.009930	0.00917	92	0.00924	93
Fortification:	Average [%]:	95			95
0.001 mg/kg	Standard deviation [%]	11.1			7.3
	Relative standard deviation [%]:	11.7			7.7
	Number of determinations:	4			4
Fortification:	Average [%]:	95			95
0.01 mg/kg	Standard deviation [%]	1.7			1.8
	Relative standard deviation [%]:	1.7			1.9
	Number of determinations:	5			5

* Malfunction of the ASE apparatus. This result was not taken into account to calculate statistical data.

The accuracy of the method is acceptable for each analyte.

LOQ
The LOQ of the method is 0.001mg/kg for each analyte.

Conclusion

The analytical method is suitable for the determination of Difenoconazole and CGA 205375 in soil by LC-MS/MS with a LOQ = 0.001mg/kg for each analyte. The method is considered as highly specific. No confirmatory method is required.

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

This study (Faessel V., 2013b) have already been submitted in Poland (zRMS) to support the authorisation of the registered product Difcor 250 EC and was positively evaluated. For completeness, the summary is given below once again.

Comments of zRMS:	According to the applicant information the study was formerly recognized as acceptable.

	The validation study has been positively evaluated in Part B Section 2 of the Registration Report from 0.7.03.2016 prepared by Eko-Futura Sp. z.0.0; IOR for the extension of authorisation of product Difcor 250 EC for minor uses. The following conclusion was made: *„To be fit for the intended purpose, the method was successfully evaluated and met certain validation characteristics: specificity, linearity, repeatability and accuracy. The analytical method fulfils the criteria of SANCO/825/00 rev. 8.1.* *The study has been performed in compliance with Good Laboratory Practice and is acceptable."*

Reference:	KCP 5.2-05
Report	Validation of the Analytical Method for the Determination of Difenoconazole Residues in ground and surface water, Faessel V., 2013b, R B2299
Guideline(s):	Yes (SANCO/825/00 rev. 8.1)
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Difenoconazole were extracted from samples using an Anadiag method:
For the preparation and extraction of the samples: SOP MP 290
For the analysis of extracts and for the calibration: SOP MA 616

A clean-up by liquid partition with dichloromethane is first made. The dichloromethane layers are then concentrated down to about 2 mL using a rotary evaporator then just to dryness under a gentle stream of nitrogen. The residue is then dissolved in 5 mL ethanol. Matrix matched calibration solutions were used for calibration. Difenoconazole is then quantified by LC with MS/MS detection: ESI, 406>251 m/z ; 406>306m/z

Results and discussions

Specificity
Chromatograms of control samples, of standard solution and of fortified samples are provided. No interference is observed at the retention time of difenoconazole. The specificity of the method is acceptable.

Linearity
The detector response for difenoconazole analysis was linear within the range from 0.15 to 6.01 ng/mL (n=7) in ground water and from 0.3 to 12 ng/mL (n=7) with $r^2 > 0.90$ in surface water.

Ground water:
Quantification: y=1.7360E-04x-0.07, r=0.99773
Confirmation: y=1.0618E-03x-0.05, r=0.99781

Surface water:
Quantification: y=1.7548E-04x-0.11, r=0.99943
Confirmation: y=1.0340E-03x-0.11, r=0.99897

The linearity of the method is acceptable

Accuracy

% RSD and % mean recoveries are reported below:

Quantification 406>251 m/z

Analyte	Matrix	Fortification level (µg/L)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	Ground water	0.05	76.5	5.5	7.1	5
		0.5	89.7	1.5	1.6	5
Difenoconazole	Surface water	0.1	94.7	2.8	3	5
		1	92.7	6.7	7.2	5

Confirmation 406>306m/z

Analyte	Matrix	Fortification level (µg/L)	Mean recovery Percentage (%)	Standard deviation (SD) (%)	Relative standard deviation (RSD) (%)	Number of fortified samples (n)
Difenoconazole	Ground water	0.05 (LOQ)	**76.4%**	**7.8%**	**10.2%**	5
Difenoconazole	Surface water	0.10 (LOQ)	**95.8%**	**4.6%**	**4.8%**	5

LOQ
The LOQ of the method is 0.1 µg/L for surface water and 0.05 µg/L for drinking water.

Conclusion

The method is suitable for determination of difenoconazole at 0.1 µg/L in surface water and at 0.05 µg/L in ground water. The method is not considered as highly specific because the confirmation (406>306m/z) has been performed on one fortification level.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

A 2.1.2.7 Other Studies/ Information