

# FINAL REGISTRATION REPORT

## **Part B**

### **Section 5**

#### **Analytical Methods**

Detailed summary of the risk assessment

Product code: **FLUDIO 025 GF**

Product names: **FLUDIO ŽEL 025 FS /**

**FUNABEN® ŽEL 025 FS**

Chemical active substance:

Fludioxonil, 25 g/L

Central Zone

Zonal Rapporteur Member State: **Poland**

**CORE ASSESSMENT**

(authorization)

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

Submission date: **01/2023**

MS Finalisation date: **06/2023, 10/2023**

## Version history

When	What
01/2023	Initial dRR
06/2023	Initial zRMS assessment
10/2023	The final Registration Report

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## 5 Analytical methods

Justification regarding the difference in the formulation type between the product code name - FLUDIO 025 GF and the product trade names - FLUDIO ŽEL 025 FS, FUNABEN® ŽEL 025 FS is presented in Part C.

The product code name FLUDIO 025 GF is used in all draft Registration Report.

### 5.1 Conclusion and summary of assessment

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

Noticed data gaps are:

- None

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

Data gaps:

- ILV method for drinking water (post registration requirement)
- Methods for body fluids and tissues (post registration requirement):

Commodity/crop	Supported/ Not supported
Cereals	Supported

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

#### 5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of zRMS:	The proposed analytical method is suitable for the determination of the active substance fludioxonil in the plant protection product Fludio Žel 025 FS. The proposed analytical method has been fully validated in terms of interference, specificity, linearity, accuracy and precision. The proposed method fulfils the requirements of SANCO/3030/99 rev.5 guidance. The validation of the analytical method has been accepted.
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Reference: Validation included in the following reports:

Jarosław Kupiec, M.Sc., 2022/ Synthos Agro

Report: Validation included in the report: **FLUDIO 025 GF**, Stage I: Determination of physicochemical properties of the initial preparation, after accelerated storage and after low temperature storage, Kupiec Jarosław, M.Sc., 2022, Study code: BF-59/21

Guideline(s): Yes, SANCO/3030/99 rev.5

Deviations: No

GLP: Yes

Acceptability: Yes

### Materials and methods

The determination of the active ingredient – fludioxonil content in FLUDIO 025 GF preparation were carried out in accordance with the method – MT/BA-01/22 – developed and validated according to EU requirements described in SANCO/3030/99 rev. 5 (22/03/1) guideline.

The method (MT/BA-01/22) is based on determination of fludioxonil using reversed phase high performance liquid chromatography (RP-HPLC) with UV-VIS detection at wavelength 265nm and external standard at the initial stage and after accelerated storage.

#### Examined material

Examined material: FLUDIO 025 GF  
Date of production: 10.2021  
Batch number: SNS-F-06-22  
Manufacturer: Synthos Agro Sp. z o.o.  
Test item code: 99/21

#### Reference material:

Fludioxonil, IPO 937, batch 2A/21, purity 99.90%

#### Apparatus and materials

- Shimadzu liquid chromatograph equipped with UV/VIS detector
- Column: Luna C18, 250 x 4.6 mm, 5µm
- Analytical balance
- Ultrasonic bath
- Volumetric flasks
- Volumetric pipette
- Syringe filters PTFE, 0,22 µm

#### Reagents

- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC-Super Gradient, POCh

#### Chromatographic conditions

- Oven temperature: 30 °C
- Flow rate: 1.4 ml/min
- Wavelength  $\lambda$  = 265 nm
- Volume injection: 5 µl

- Mobile phase composition: acetonitrile + H<sub>2</sub>O (53 + 47, v/v)

Under the above conditions the retention time of Fludioxonil is about  $9.1 \pm 0.3$  min. and the total time of analysis is 20.0 min.

## Preparation of solutions

### Standards solutions

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

Fludixonil – standard, purity = 99.9%		
Chromatogram name	Mass [mg]	C [mg/ml]
wz1-1,1-2	5.30	0.529
wz2-1,2-2	5.90	0.589

### Specimen solutions

About 200 mg of examined specimen was weighed (with the accuracy of 0.01 mg) into a 10 ml flask. 2 ml water and acetonitrile was added, stirred and the flask was put into the ultrasonic bath for 5 min. After cooling, acetonitrile was added up to the nominal volume. Solutions of examined sample were passed through syringe filters and analyzed.

## Validation - Results and discussions

**Table 5.2-1: Methods suitable for the determination of active substance Fludioxonil in plant protection product FLUDIO 025 GF**

	Fludioxonil
<b>Author(s), year</b>	Jarosław Kupiec, M.Sc., 2022
<b>Principle of method</b>	The determination of the active ingredient – Fludioxonil content in FLUDIO 025 GF preparation were carried out in accordance with the method – MT/BA-01/22 – developed and validated according to EU requirements described in SANCO/3030/99 rev. 5 (22/03/1) guideline. The method (MT/BA-01/22) is based on determination of fludioxonil using reversed phase high performance liquid chromatography (RP-HPLC) with UV-VIS detection at wavelength 265nm and external standard at the initial stage and after accelerated storage.
<b>Linearity</b> <b>Linear between:</b> <b>0.3531 mg/ml to 0.6558 mg/ml</b>	The linearity of the detector response was assessed using five standards solutions of fludioxonil in the concentration range from 0.3531 mg/ml to 0.6558 mg/ml. To prepare the calibration curve volumes of: 0.70 ml, 0.80 ml, 1.00 ml, 1.20 ml and 1.30 ml of standard solution (5.0450 mg/ml) were pipetted to 10 ml flasks and acetonitrile was added to the nominal volume. $y = 9,951,739 x + 22,543$ $R^2 = 0.999$ Correlation coefficient should be $r \geq 0.99$ . The obtained result is acceptable.
<b>Precision – Repeatability Mean</b> <b>n = 6</b> <b>1.51 %RSD</b>	The content of fludioxonil in the FLUDIO 025 GF preparation was determined by analysis of six - about 200 mg - portions of the specimen solution.  Acceptable relative standard deviation for analyte in preparation (~

	Fludioxonil
	2.41%) is $RSDr \leq 2.35\%$ . The obtained result $RSDr = 1.51\%$ and the Horrat value $Hr = 0.64$ is acceptable.
<b>Accuracy</b> <b>n = 12</b> <b>100.22 % Recovery</b>	Recovery of the method determination of Fludioxonil content in FLUDIO 025 GF preparation was assessed by total recovery. To twelve 5 ml flasks, 1 ml placebo of FLUDIO 025 GF was added. To six of them 0.50 ml (level I; concentration of Fludioxonil standard solution – 4.577 mg/ml) and to other six 0.70 ml (level II; concentration of Fludioxonil standard solution – 4.129 mg/ml) of Fludioxonil standard solution were added. Acetonitrile was added up to the mark and the mixture was put into an ultrasonic bath for 5 minutes. Solutions were passed through syringe filters and analyzed.  The result of 100.22% fulfils the acceptance criterion (90 – 110%).
<b>Interference/ Specificity</b>	To prove specificity of the developed method, chromatograms of: solvent, standard, placebo and specimen of FLUDIO 025 GF were performed and superimposed. There are no other peaks that could interfere with peak of fludioxonil under the specified chromatographic conditions.
<b>Comment</b>	The validation parameters (specificity, linearity, LOQ, repeatability and accuracy) are within the acceptance range and fulfil EU requirements given in SANCO /3030 /99 rev.5. There seems to be an error in the report in the part regarding the recovery: for level I, 0.5 ml of fludioxonil standard (4.577 mg/mL) was diluted in 5 mL giving the concentration 0.4577 mg/mL of fludioxonil added whereas in the report the value of 0.4557 mg/mL is given. However, this has minimal impact on the recovery value calculated (100.22% vs 101.14%) and RSD (0.91% in both cases) so it can be accepted.

## Conclusion

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

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

No analytical methods for determination of the relevant impurities were developed for the formulated product since no toxicologically or eco-toxicologically relevant impurities are expected to be formed during the formulation process.

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

With respect to toxicological, eco-toxicological or environmental aspects FLUDIO 025 GF does not contain any relevant formulants. Therefore, a special analytical method and validation is not needed.

#### 5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

There are no CIPAC methods available for determination of fludioxonil

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

**Table 5.2-2: Validated methods for the generation of pre-authorization data**

Component of residue definition: Fludioxonil				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Wheat grains Wheat straw (Residues)	Primary	0.01 mg/kg Wheat straw 0.01 mg/kg Wheat grains	LC-MS/MS	Zaręba-Kozioł M./ 2022 Synthos Agro Wójcik M./ 2022, Synthos Agro
	Confirmatory (if required)	Not required due to specific method to the analytes. According to SANCO/3029/99 rev. 5		
Soil – Silty clay, Sandy loam (Environmental fate)	Primary	0.02 mg/kg	HPLC-UV	Tribolet, R., 2001, DAR of Fludioxonil (January 2005)
	Confirmatory (if required)	No confirmatory method was provided		
Soil – Silty clay loam, Sandy loam (Environmental fate)	Primary	0.01 mg/kg	HPLC-MS/MS	Tribolet, R., 2001, DAR of Fludioxonil (January 2005)
	Confirmatory (if required)	Only one transition was monitored, the method cannot be highly specific but it was discussed in the PRAPeR 06 expert meeting		
Soil (Ecotoxicology)	Primary	LOQ 2.5 mg/kg	HPLC-DAD	Wróbel A., 2022/ Synthos Agro Gierbuszewska A., 2022/ Synthos Agro
	Confirmatory (if required)	Not required due to specific method to the analytes. According to SANCO/3029/99 rev. 5		
Water (Ecotoxicology)	Primary	LOQ 0.0005 mg/L	HPLC-DAD	Hodorek G., 2022/ Synthos Agro Nierzędska E., 2022/ Synthos Agro
	Confirmatory (if required)	Not required due to specific method to the analytes. According to SANCO/3029/99 rev. 5		



<b>Component of residue definition: The sum of Fludioxonil and its metabolites oxidized to metabolite CGA192155</b>				
<b>Matrix type</b>	<b>Method type</b>	<b>Method LOQ</b>	<b>Principle of method (i.e. GC-MS or HPLC-UV)</b>	<b>Author(s), year / missing / EU agreed</b>
Animal products, food of animal origin (Residues)	Primary	0.01 mg/kg in muscles and milk 0.05 mg/kg in liver, kidney and fat	HPLC-UV	<b>AG 616</b> Vienneau K., 1996, DAR of Fludioxonil (January 2005) Annex B.5 Analytical methods; Point B.5.2.2.1  Tang j., Baldi B., 1996, DAR of Fludioxonil (January 2005) Annex B.5 Analytical methods; Point B.5.2.2.1
	Confirmatory (if required)	Not required due to specific method to the analytes. According to SANCO/3029/99 rev. 4 Validation is available in the monitoring part performed in ILV.		

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

#### 5.3.1 Analysis of the plant protection product (KCP 5.2)

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

#### 5.3.2 Description of analytical methods for the determination of residues of Fludioxonil (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**

<b>Matrix</b>	<b>Residue definition</b>	<b>MRL / limit</b>	<b>Reference for MRL/level Remarks</b>
Plant, high water content	Fludioxonil	0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Plant, high acid content		0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Plant, high protein/high starch content (dry)		0.01 mg/kg	Regulation (EU) No 2022/1264 Annex

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
commodities)			
Plant, high oil content		0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg	Regulation (EU) No 2022/1264 Annex
Muscle	Fludioxonil	0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Milk		0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Eggs		0.02 mg/kg	Regulation (EU) No 2022/1264 Annex
Fat		0.01 mg/kg	Regulation (EU) No 2022/1264 Annex
Liver, kidney		0.1 mg/kg	Regulation (EU) No 2022/1264 Annex
Soil (Ecotoxicology)	Fludioxonil	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Fludioxonil	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Fludioxonil	18 µg/L	NOEC Daphna magna; EFSA Scientific Report (2007) 110, 1-85
Air	Fludioxonil	Not required 2 µg/m <sup>3</sup>	Not classified as T / T+ EFSA, 2007
Tissue (meat or liver)	Fludioxonil	Not required 0.01 mg/kg	Not classified as T / T+ Default LOQ SANTE/2020/12830, Rev.1
Body fluids		Not required 0.01 mg/kg	Not classified as T / T+ Default LOQ SANTE/2020/12830, Rev.1

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

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

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

Component of residue definition: Fludioxonil				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg	HPLC-MS/MS	Lakaschus, S., 2005, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	ILV	0.01 mg/kg	HPLC-MS/MS	Reichert N., 2006, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary. Two transitions were monitored.		
High acid content	Primary	0.01 mg/kg	HPLC-MS/MS	Lakaschus, S., 2005, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	ILV	0.01 mg/kg (for Avocado and Kiwi)	HPLC-MS/MS	Reichert N., 2006, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary. Two transitions were monitored.		
High oil content	Primary	0.01 mg/kg	HPLC-MS/MS	Lakaschus, S., 2005, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	ILV	0.01 mg/kg	HPLC-MS/MS	Reichert N., 2006, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary. Two transitions were monitored.		
High protein/high starch content (dry)	Primary	0.01 mg/kg	HPLC-MS/MS	Lakaschus, S., 2005, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	ILV	0.01 mg/kg (for Avocado and Kiwi)	HPLC-MS/MS	Reichert N., 2006, Addendum to the DAR of Fludioxonil (October 2006, B.5)
	Confirmatory (if required)	The original method is highly specific therefore an additional confirmatory method is not necessary. Two transitions were monitored.		
Difficult (if required, depends on intended use)	Primary	Not required. The product is intended to use as a cereals seed treatment.		
	ILV			
	Confirmatory (if required)			

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

**Table 5.3-3: Statement on extraction efficiency**

	Method for products of plant origin
Required, available from:	DAR of Fludioxonil (October 2006, B.5.2.2.1; Mair, 1996)
Not required, because:	

For the detailed evaluation of (additional) studies on extraction efficiency, it is referred to Appendix 2.

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

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

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

Component of residue definition: Fludioxonil and its metabolite CGA192155				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Milk	Primary	0.01 mg/kg	HPLC-UV	Vienneau K., 1996, DAR of Fludioxonil (January 2005, B.5)
	ILV	0.01 mg/kg	HPLC-UV	Tang j., Baldi., 1996 DAR of Fludioxonil (January 2005, B.5)
	Confirmatory (if required)	A confirmatory method was provided according to DAR of Fludioxonil (January 2005, B.5)		
Eggs	Primary	0.05 mg/kg	HPLC-UV	Vienneau K., 1996, DAR of Fludioxonil (January 2005, B.5)
	ILV	0.05 mg/kg	HPLC-UV	Tang j., Baldi., 1996 DAR of Fludioxonil (January 2005, B.5)
	Confirmatory (if required)	A confirmatory method was provided according to DAR of Fludioxonil (January 2005, B.5)		
Muscle	Primary	0.01 mg/kg	HPLC-UV	Vienneau K., 1996, DAR of Fludioxonil (January 2005, B.5)
	ILV	ILV method was provided for Milk, Liver and Eggs matrix according to DAR of Fludioxonil (January 2005, B.5)		
	Confirmatory (if required)	A confirmatory method was provided but not validated according to DAR of Fludioxonil (January 2005, B.5)		
Fat	Primary	0.05 mg/kg	HPLC-UV	Vienneau K., 1996, DAR of Fludioxonil (January 2005, B.5)
	ILV	ILV method was provided for Milk, Liver and Eggs matrix according to DAR of Fludioxonil (January 2005, B.5)		
	Confirmatory (if required)	A confirmatory method was provided but not validated according to DAR of Fludioxonil (January 2005, B.5)		
Kidney, liver	Primary	0.05 mg/kg	HPLC-UV	Vienneau K., 1996, DAR of Fludioxonil (January 2005, B.5)

Component of residue definition: Fludioxonil and its metabolite CGA192155				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
	ILV	0.05 mg/kg	HPLC-UV	Tang j., Baldi., 1996, DAR of Fludioxonil (January 2005, B.5)
	Confirmatory (if required)	ILV method was provided for Milk, Liver and Eggs matrix according to DAR of Fludioxonil (January 2005, B.5)		

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:	Vienneau, K. P., 1996 DAR of Fludioxonil (January 2005, B.5), available in RAR of Fludioxonil (February 2018, B.5)
Not required, because:	-

For the detailed evaluation of (additional) studies on extraction efficiency please refer to Appendix 2.

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

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

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

Component of residue definition: Fludioxonil			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg	HPLC-MS-MS	Robinson N.J., Tummon O.J., 2004, DAR of Fludioxonil (January 2005, B.5)
Confirmatory	Only one transition was monitored. The method cannot be highly specific according to DAR of Fludioxonil (January 2005, B.5)		
Primary	0.02 mg/kg	HPLC-UV	Tribolet R., 2001, DAR of Fludioxonil (January 2005, B.5)
Confirmatory	No confirmatory method was provided according to DAR of Fludioxonil (January 2005, B.5)		

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 fludioxonil in surface and drinking water is given in the following tables. For the detailed valuation of new/ additional studies it is referred to Appendix 2.

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

Component of residue definition: Fludioxonil				
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	HPLC-UV	Tribolet R., 1999, DAR of Fludioxonil (January 2005, B.5)
	ILV	No ILV method was provided according to DAR of Fludioxonil (January 2005, B.5)		
	Confirmatory	No confirmatory method was provided according to DAR of Fludioxonil (January 2005, B.5)		
Surface water	Primary	0.1 µg/L	HPLC-UV	Tribolet R., 1999, DAR of Fludioxonil (January 2005, B.5)
	Confirmatory	No confirmatory method was provided according to DAR of Fludioxonil (January 2005, B.5)		

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

#### **zRMS:**

Data gap: ILV method for drinking water.

### 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 fludioxonil in air is given in the following tables. For the detailed evaluation of new/ additional studies please refer to Appendix 2.

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

Component of residue definition: Fludioxonil			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	2 µg/m <sup>3</sup>	HPLC-UV	Tribolet R., 1992, DAR of Fludioxonil (January 2005, B.5)
Confirmatory	No confirmatory method is required, since method is highly specific.		
Primary	2 µg/m <sup>3</sup>	HPLC-UV	Tribolet R., 1992, DAR of

Component of residue definition: Fludioxonil			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
			Fludioxonil (January 2005, B.5)
Confirmatory	No confirmatory method is required, since method is highly specific.		
Primary	2 µg/m <sup>3</sup>	HPLC-UV	Tribolet R., 1992, DAR of Fludioxonil (January 2005, B.5)
Confirmatory	No confirmatory method is required, since method is highly specific.		

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

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

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

**Table 5.3-9: Methods for body fluids and tissues (if appropriate)**

Component of residue definition: Fludioxonil			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	Not required, since substance is not classified as toxic (T) or very toxic (T <sup>+</sup> )		
Confirmatory	Not required, since substance is not classified as toxic (T) or very toxic (T <sup>+</sup> )		

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 2.

#### **zRMS:**

Methods are required (according to REGULATION (EU) No 284/2013)

#### 5.3.2.8 Other studies/ information

No other studies or information is provided.

## Appendix 1 Lists of data considered in support of the evaluation

### List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1	Kupiec Jarosław, M.Sc.	2022	FLUDIO 025 GF. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated storage and after low temperature storage. Jarosław Kupiec, M.Sc., 2022, Study code: BF-59/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wójcik, M. MSc Eng.	2022	DIFLUD 050 FS Determination of the residues of difenoconazole, triazole derivative metabolites and fludioxonil in grains and straw of winter wheat. Marcin Wójcik, MSc Eng, 2022 Study code: C-02-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Zaręba-Kozioł, M. PhD	2022	Validation of a method for determination of fludioxonil and its metabolite CGA192155 residues by Liquid Chromatography (LC-MS/MS) Monika Zaręba-Kozioł, PhD, 2022 Study code: PW-2022-02 Fertico Sp. z o.o. GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wróbel A., MSc	2022	FLUDIO 025 GF Earthworm ( <i>Eisenia andrei</i> ) reproduction test. Anna Wróbel, MSc, 2022	N	Synthos Agro Sp. z o.o.



Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Study code: G/23/21 GLP Unpublished		
KCP 5.2	Gierbuszewska, A., MSc	2022	FLUDIO 025 GF Collembolan ( <i>Folsomia candida</i> ) Reproduction Test. Aneta Gierbuszewska, MSc, 2022 Study code: G/24/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Wróbel A., MSc	2022	FLUDIO 025 GF Predatory mite ( <i>Hypoaspis (Geolaelaps) aculeifer</i> ) reproduction test in soil. Anna Wróbel, MSc, 2022 Study code: G/25/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Hodorek G., MSc	2022	FLUDIO 025 GF Daphnia magna, Acute Immobilisation Test. Grażyna Hodorek, MSc, 2022 Study code: W/31/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Hodorek G., MSc	2022	FLUDIO 025 GF <i>Raphidocelis subcapitata</i> SAG 61.81 (formerly <i>Pseudo-kirchneriella subcapitata</i> ), Growth inhibition test. Grażyna Hodorek, MSc, 2022 Study code: W/32/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	■■■■	2022	FLUDIO 025 Rainbow trout, Acute Toxicity Testing. ■■■■	N	Synthos Agro Sp. z o.o.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Study code: W/33/21 █ GLP Unpublished		
KCP 5.2	Kulec-Płoszczyca, E., MSc	2022	FLUDIO 025 GF Bumblebees ( <i>Bombus spp.</i> ), Acute Oral Toxicity Test. Elżbieta Kulec-Płoszczyca, MSc, 2022 Study code: B/67/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Kulec-Płoszczyca, E., MSc	2022	FLUDIO 025 GF Bumblebees ( <i>Bombus spp.</i> ), Acute Contact Toxicity Test. Elżbieta Kulec-Płoszczyca, MSc, 2022 Study code: B/68/21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Kulec-Płoszczyca, E., MSc	2022	FLUDIO 025 GF Honeybees ( <i>Apis mellifera L.</i> ), Larval Toxicity Test, Repeated Exposure. Elżbieta Kulec-Płoszczyca, MSc, 2022 Study code: B/01/22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.
KCP 5.2	Kulec-Płoszczyca, E., MSc	2022	FLUDIO 025 GF Honeybees ( <i>Apis mellifera L.</i> ), Chronic Oral Toxicity Test. Elżbieta Kulec-Płoszczyca, MSc, 2022 Study code: B/02/22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry Branch Pszczyna GLP Unpublished	N	Synthos Agro Sp. z o.o.

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

<b>Data point</b>	<b>Author(s)</b>	<b>Year</b>	<b>Title Company Report No. Source (where different from company) GLP or GEP status Published or not</b>	<b>Vertebrate study Y/N</b>	<b>Owner</b>
KCP 5.1.2	Mair P.	1993	Determination of CGA 173506 in plant material, wine and soil by HPLC incl. validation data. NCP/Novartis Crop Protection AG, -, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No REM-133-04, 01/04/1993 Syngenta File N° CGA173506/0313 GLP Not Published	N	Syngenta
KCP 5.1.2	Mair P.	1996	Validation of Method REM 133.04. Determination of efficiency of extraction and accountability from 14C-Fludioxonil treated specimen (tomato) Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland Report No 110/96 Syngenta File No CGA173506/0778 GLP Not Published	N	Syngenta
KCP 5.1.2	■■■■	1996	Independent Method Validation Ruggedness Trial for the Determination of Total Residues of CGA173506 and Metabolites in Animal Tissues, Milk and Eggs using Ciba-Geigy Method AG-616B entitled “Determination of Total Residues of CGA1735...” Novartis Crop Protection AG, Basel, Switzerland ■■■■ Syngenta File No CGA173506/0886 GLP Not Published	N	Syngenta
KCP 5.1.2	Tribolet R.	2001	Validation of Method REM 133.04 by Analysis of Fortified Specimens (Plant Materials and Soil) for Fludioxonil (CGA173506) and Evaluation of Recoveries Syngenta Crop Protection AG, Basel, Switzerland Report No 210/01, 26/06/2001 Syngenta File N° CGA173506/5398 GLP Not Published	N	Syngenta

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2	■■■■	1996	Determination of total residues of CGA173506 and metabolites as CGA192155 in animal tissues, milk and eggs by high performance liquid chromatography with column switching Novartis Crop Protection AG, Basel, Switzerland ■■■■ Report No AG-616B Syngenta File No CGA173506/0733 GLP Not Published	N	Syngenta
KCP 5.2	Lakaschus, S.	2005	Validation of Multi-Residue Method DFG S19 (L00.00-34) for the Determination of Residues of Fludioxonil in Different Plant Matrices With LC-MS/MS Detection Syngenta Crop Protection AG, Basel, Switzerland Dr. Specht & Partner Chem. Laboratorien GmbH, Hamburg, Germany Method/Validation No. SYN-0503V, 01 August 2005 GLP, Not Published Syngenta File N° CGA173506/6497	N	Syngenta
KCP 5.2	Mair, P.	1996	Validation of Method REM 133.04. Determination of efficiency of extraction and accountability from 14C-Fludioxonil treated specimen (tomato) Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland Report No 110/96 GLP Not Published Syngenta File No CGA173506/0778	N	Syngenta
KCP 5.2	Reichert N.	2006	Independent Laboratory Validation of the DFG Method S19 for The Determination of Residues of Fludioxonil in Plant Matrices (Kiwi and Avocado) Syngenta Crop Protection AG, Basel, Switzerland Institut Fresenius, Taunusstein, Germany Method/Validation No. IF-05/00362984, 26 January 2006 GLP Not Published Syngenta File N° CGA173506/6772	N	Syngenta

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	Robinson N.J., Tummon O.J.	2004	Residue Analytical Method for the Determination of Fludioxonil in Soil Syngenta Ltd., Jealott's Hill, UK, 02.07.2004 Study report number RAM 423/01 Not GLP Not Published Syngenta archive No. CGA173506/5941	N	Syngenta
KCP 5.2	■■■■■	1996	Independent Method Validation Ruggedness Trial for the Determination of Total Residues of CGA173506 and Metabolites in Animal Tissues, Milk and Eggs using Ciba-Geigy Method AG-616B entitled "Determination of Total Residues of CGA1735..." Novartis Crop Protection AG, Basel, Switzerland ■■■■■ Report No 96-0010 GLP Not Published Syngenta File No CGA173506/0886	N	Syngenta
KCP 5.2	Tribolet R.	1992	Sampling of air and determination of residues of parent compound by high performance liquid chromatography NCP/Novartis Crop Protection AG, Switzerland Ciba-Geigy Ltd., Basel, Switzerland Report No REM 133-03, 15/12/1992 GLP Not Published Syngenta File N° CGA173506/0234	N	Syngenta
KCP 5.2	Tribolet R.	2001	Validation of Method REM 133.04 by Analysis of Fortified Specimens (Plant Materials and Soil) for Fludioxonil (CGA173506) and Evaluation of Recoveries Syngenta Crop Protection AG, Basel, Switzerland Report No 210/01, 26/06/2001 GLP Not Published Syngenta File N° CGA173506/5398	N	Syngenta

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	■■■■	1996	Validation of “Draft” Analytical Method AG-616 for the Determination of Total Residues of CGA173506 and Metabolites as CGA192155 in Animal Tissues, Milk and Eggs Novartis Crop Protection AG, Basel, Switzerland ■■■■ Report No ABR-95063 GLP Not Published Syngenta File No CGA173506/0732	N	Syngenta
KCP 5.2	■■■■	1996	Determination of total residues of CGA173506 and metabolites as CGA192155 in animal tissues, milk and eggs by high performance liquid chromatography with column switching Novartis Crop Protection AG, Basel, Switzerland ■■■■ Report No AG-616B Not GLP Not Published Syngenta File No CGA173506/0733	N	Syngenta

## Appendix 2 Detailed evaluation of submitted analytical methods

### A 2.1 Analytical methods for the Fludioxonil

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

No new or additional studies have been submitted

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

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

###### A 2.1.2.1.1 Analytical method 1

###### A 2.1.2.1.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Marcin Wójcik, 2022/ Synthos Agro

Report Validation included in the report: DIFLUD 050 FS Determination of the residues of difenoconazole, triazole derivative metabolites and fludioxonil in grains and straw of winter wheat, Marcin Wójcik, MSc Eng., 2022, Study code: C-02-22

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

Deviations: No

GLP: Yes

Acceptability: Yes

### Materials and methods

The analytical method was developed for the determination of fludioxonil and its metabolite (CGA 192155) in grains and straw of winter wheat. The validation was involved the assessment of the linearity of response of the analytical graph, specificity, precision, accuracy, matrix effects and limits of quantification and detection. Moreover the chromatographic system and conditions chemical analysis were set. The validation of analytical method was performed according to SANTE/2020/12830 rev.1

### Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution with a concentration of 1.0 mg/mL was prepared for fludioxonil and CGA 192155 individually by weighting 10.0 mg of standards into a volumetric flask with a capacity of 10 mL, dissolving in acetonitrile for LC-MS, and next the volume was made up to 10 ml with the same solvent. Intermediate standard solutions of standards mixture at concentration 10 µg/mL and 1.0 µg/mL were prepared by dilution of stock solutions with acetonitrile for LC-MS.

The working solutions were prepared by diluting standards with a higher concentration. The dilutions were made as exemplarily described in the table below:

Take solution at concentration [ng/mL]	Aliquot Volume [mL]	Dilute with D1 solution to a final volume of [mL]	Fina Concentration [ng/mL]
1000	0.1	1	100
1000	0.05	1	50 <sup>1)</sup>
100	0.2	1	20 <sup>1)</sup>
100	0.1	1	10 <sup>1)</sup>
50	0.1	1	5 <sup>1)</sup>
20	0.1	1	2 <sup>1)</sup>
10	0.1	1	1 <sup>1)</sup>
5	0.1	1	0.5 <sup>1)</sup>

1) Concentration level used for calibration.

D1 – mixture of acetonitrile : formic acid LC-MS (1000 : 1, v/v)

### Preparation of Fortified Sample

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

The following fortification scheme was used:

Matrix	Sample Type	Number of repetitions	Sample Weight [g]	Concentration Of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Winter wheat grains	Blank matrix	2	5.0	-	-	0.0
	Fortification (LOQ)	5	5.0	1.0	0.05	0.01
	Fortification (10x LOQ)	5	5.0	10.0	0.05	0.10
Winter wheat straw	Blank matrix	2	2.0	-	-	
	Fortification (LOQ)	5	2.0	1.0	0.02	0.01
	Fortification (10x LOQ)	5	2.0	10.0	0.02	0.10

Fortification samples were prepared and analysed to ensure the result fits within the range of the respective standard curve.

### Sample preparation for the chromatographic analysis

#### **Grains**

First, 5 g ground (frozen) grains of winter wheat was weighed in a 50 mL centrifuge tube, and next 10 mL of deionized water and 10 mL of acetonitrile was added. The sample was shaken vigorously for 1 min. by



hand. QuEChERS BEKOLut Citrate-Kit-01 was added to the sample mixture and shaken vigorously for 0.5 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and decanted. QuEChERS BEKOLut PSA-Kit-04 was added to the aliquot and shaken vigorously for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and filtered through anhydrous sodium sulphate (VI). The volume of clear extract was made up to 25 mL with mixture of acetonitrile : formic acid (1000:1, v/v). Finally, 1.0 µL of the sample was introduced into a LC-MS column.

### Straw

First, 2 g ground (frozen) straw of winter wheat was weighed in a 50 mL centrifuge tube, and next 5 mL of deionized water and 10 mL of acetonitrile was added. The sample was shaken vigorously for 1 min. by hand. QuEChERS BEKOLut Citrate-Kit-01 was added to the sample mixture and shaken vigorously for 0.5 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and decanted. QuEChERS BEKOLut PSA-Kit-08 was added to the aliquot and shaken vigorously for 1 min. by hand. The sample was centrifuge for 5 min. at 3800 rpm and filtered through anhydrous sodium sulphate (VI). The volume of clear extract was made up to 10 mL with mixture of acetonitrile : formic acid (1000:2, v/v). Finally, 1.0 µL of the sample was introduced into a LC-MS column.

### Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SA TE/2020/12830 rev.1

### Conditions of the chemical determinations

#### Reagents and solvents

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Acetonitrile	LC-MS	VWR Chemicals	22E044007	29.04.2025
Formic acid	≥99% for LC- MS	VWR Chemicals	PW743391	31.08.2024
Sodium sulphate(VI) anhydrous	Pure 99.4%	J.T. Baker	2106706810	08.03.2026
QuEChERS BEKOLut Citrate-Kit-01	-	BEKOLut GmbH & Co. KG	4821	01.12.2024
QuEChERS BEKOLut PSA-Kit-04	-	BEKOLut GmbH & Co. KG	4621	19.11.2024

\* The main stages of water purification: pre-treatment (pre-filtration, activated carbon, polyphosphates), reverse osmosis, electro deionization, UV lamp emitting radiation with a wavelength of 254 nm. Water prepared with Elix® Essential 10 (UV) system

The following solutions were also prepared:

- mixture of acetonitrile : formic acid (1000:1, v/v).

#### Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Balance	PS 600.X2.	Radwag (Poland)
Balance	WPS 510/C	Radwag (Poland)
Volumetric flasks	Various volumes	Glassco (Germany)

Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Glass pipettes	Various volumes	Brand (Germany)
Low temperature freezer	ULUF 450	ARCTIKO (Denmark)
Blender	HGB55E	Waring Commercial (USA)
Laboratory centrifuge	MPW-351e	MPW Med. Instruments
Water purification system	Elix® Essential 10 (UV)	Merck Millipore
Filter paper	90 mm	Chemland (Poland)
Autosampler vials with PTFE/silicone septa and screw caps	Clear glass, 2 mL	Alwsci Technologies (China)
Liquid chromatograph with mass spectrometer	NEXERA XR LCMS-8045	Shimadzu Corp. (Japan)

### Conditions of analysis

The chromatographic, detection system and parameters were used for the analysis of fludioxonil and CGA 192155 are shown in the table below.

### Chromatographic System

Analytical Column

Column temperature

Injection Volume

Mobile Phase A

Mobile Phase A

Flow Rate

Gradient (including wash and equilibration)

### Parameter

Shimadzu Nexera XR

Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm

35°C

5 µL

Water

Acetonitrile / Formic acid (1000:1, v/v)

0.50 mL/min

Time [min]	Phase A [%]	Phase B [%]
0.00	90	10
0.75	90	10
1.50	5	95
2.00	5	95
2.05	90	10
4.00	90	10

### Detection System

Ionisation

### Analyte

Fludioxonil

CGA 192155

Shimadzu LCMS-8045 Mass Spectrometer

Mass Spectrometer Ionisation Electro Spray (ESI)

### Transitions

### Polarity

247.10 > 180.00<sup>1)</sup>

247.10 > 125.95<sup>2)</sup>

200.90 > 91.10<sup>1)</sup>

200.90 > 156.95<sup>2)</sup>

negative

negative

<sup>1)</sup> Quantitation transition. Mass transition used for quantification.

<sup>2)</sup> Confirmatory transition. The second transition has been monitored but will not reported, except for the validation experiment.

The same chromatographic systems and conditions will be use to the final analysis of concentration fludi-oxonil and CGA 192155 in winter wheat during the experiment.

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%) Quantification Ion	RSD (%)	Mean recovery (%) Confirmatory Ion	RSD (%)	Comments
winter wheat grains	Fludioxonil	0.01	104.5	5.5	104.5	3.7	-
		0.10	99.3	1.5	101.1	2.2	-
	CGA 192155	0.01	97.4	1.1	95.6	1.3	-
		0.10	97.7	1.3	98.0	1.3	-
winter wheat straw	Fludioxonil	0.01	98.3	5.5	96.2	7.1	-
		0.10	94.6	1.4	96.8	2.6	-
	CGA 192155	0.01	98.8	2.2	94.8	2.6	-
		0.10	97.6	1.0	95.6	1.2	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in plant matrices**

	<b>Fludioxonil</b>																		
Specificity	The analytical methods specificity were estimated on the basis of the analysis of the chromatograms obtained for the control samples, and fortified samples. Clearly labelled chromatograms of standard at the lowest calibrated levels, matrix blanks and sample fortified at the lowest fortification levels for fludioxonil and CGA 192155 for grains and straw were recorded to prove selectivity of the method.																		
Calibration (type, number of data points)	<p>The analytical calibration extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical solutions. A single determinations at 7 concentrations were made. The analytical calibration cover a two orders of magnitude.</p> <p>Working solutions of test item at the concentrations of 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL 20 ng/mL and 50 ng/mL were injected successively to the chromatographic column and the chromatograms were recorded.</p>																		
Calibration range	<p>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept), with the linear coefficient <math>r^2</math> higher than 0.99.</p> <p>Parameters of the calibration curve for winter wheat analytical method</p> <table><tr><th>Matrix</th><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td rowspan="3">Winter wheat grains</td><td>fludioxonil 247.10 &gt;180.00<sup>1</sup></td><td>5446.549</td><td>408.2036</td><td>0.9982738</td></tr><tr><td>fludioxonil 247.10 &gt;125.95<sup>2</sup></td><td>3771.638</td><td>131.4293</td><td>0.9981950</td></tr><tr><td>CGA 192155 200.90 &gt;91.10<sup>1</sup></td><td>16355.47</td><td>799.3710</td><td>0.9993920</td></tr></table>	Matrix	Analyte	Slope	Intercept	Coefficient	Winter wheat grains	fludioxonil 247.10 >180.00 <sup>1</sup>	5446.549	408.2036	0.9982738	fludioxonil 247.10 >125.95 <sup>2</sup>	3771.638	131.4293	0.9981950	CGA 192155 200.90 >91.10 <sup>1</sup>	16355.47	799.3710	0.9993920
Matrix	Analyte	Slope	Intercept	Coefficient															
Winter wheat grains	fludioxonil 247.10 >180.00 <sup>1</sup>	5446.549	408.2036	0.9982738															
	fludioxonil 247.10 >125.95 <sup>2</sup>	3771.638	131.4293	0.9981950															
	CGA 192155 200.90 >91.10 <sup>1</sup>	16355.47	799.3710	0.9993920															

	Fludioxonil																
		CGA 192155 200.90 > 156.95 <sup>2</sup>	12468.11	1252.090	0.9989731												
	Winter wheat straw	fludioxonil 247.10 >180.00 <sup>1</sup>	5613.335	- 99.73812	0.9998256												
		fludioxonil 247.10 > 125.95 <sup>2</sup>	3809.092	282.9086	0.9998741												
		CGA 192155 200.90 > 91.10 <sup>1</sup>	18350.34	- 1417.557	0.9998952												
		CGA 192155 200.90 > 156.95 <sup>2</sup>	14340.03	1201.143	0.9990895												
	<sup>1</sup> Quantification Ion <sup>2</sup> Confirmatory Ion																
Assessment of matrix effects is presented	yes																
Limit of determination/quantification	<p>Limit of Quantification is defined as the lowest validated level with sufficient recovery and precision (70 – 120% with a relative standard deviation of equal to or lower than 20%).</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample. The LoD was expressed as lowest calibration standard.</p> <p>Limit of quantification and a limit of detection for winter wheat grains and straw are presented in the table below.</p> <table><tr><th>Detected substance</th><th>Matrix</th><th>LOQ [mg/kg]</th><th>LOD [mg/kg]</th></tr><tr><td>fludioxonil</td><td>Winter wheat (grains, straw)</td><td>0.01</td><td>0.0025</td></tr><tr><td>CGA 192155</td><td>Winter wheat (grains, straw)</td><td>0.01</td><td>0.0025</td></tr></table>					Detected substance	Matrix	LOQ [mg/kg]	LOD [mg/kg]	fludioxonil	Winter wheat (grains, straw)	0.01	0.0025	CGA 192155	Winter wheat (grains, straw)	0.01	0.0025
Detected substance	Matrix	LOQ [mg/kg]	LOD [mg/kg]														
fludioxonil	Winter wheat (grains, straw)	0.01	0.0025														
CGA 192155	Winter wheat (grains, straw)	0.01	0.0025														

## Conclusion

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

### A 2.1.2.1.2 Analytical method 2

#### A 2.1.2.1.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	Monika Zaręba-Kozioł, 2022/ Synthos Agro
Report	Validation of a method for determination of fludioxonil and its metabolite CGA192155 residues by Liquid Chromatography (LC-MS/MS), Monika Zaręba-Kozioł, PhD, 2022, Study code: PW-2022-02
Guideline(s):	SANTE/2020/12830 rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

## Materials and methods

The study in the analytical phase involves a quantitative analysis of fludioxonil and CGA192155. All analyses were carried out using liquid chromatography coupled with mass spectrometry (LC-MS/MS). Analysis were performed on the winter wheat (grain and straw separately) matrix. The limit of quantification (LOQ) for fludioxonil and CGA192155 is 0.01 mg/kg. The limits of quantification were determined as a result of method validation.

### Preparation of analytical standards

The analytical standards of fludioxonil and CGA192155 were weighed into a 10 ml single-measuring vial and its solution was prepared at a concentration of 10 µg/ml, in acetonitrile, as follows:

- 1) 10.00 ± 0.01 mg of analyte standard was dissolved in 10 ml of acetonitrile (10 ml single-measuring flask) to give a concentration of R = 1000 µg/ml,
- 2) from solution R, 10 ml of standard solution R0 at 10 µg/ml was prepared.
- 3) from R0, R1 solution at 1 µg/ml was prepared
- 4) from R1 R2 solution at 0,1 µg/ml was prepared
- 5) from R2 R3 solution at 0,01 µg/ml was prepared

Stock Solution [µg /ml]	Volume taken from stock solution [µl]	Final volume [µl]	Final concentration [µg/ml]
R=1000	2x40	4000	10
R0=10	1600		4
R0=10	800		2
R0=10	400		1
R1=1	1600		0,4
R1=1	800		0,2
R1=1	400		0,1
R2=0,1	1600		0,04
R2=0,1	800		0,02
R2=0,1	400		0,01
R3=0,01	1600		0,004
R3=0,01	800		0,002

### Preparation of samples for validation

Untreated homogenous matrix samples were weigh at 5 g +/- 0.05 (grain) and 2,5 g +/- 0.025 (straw) into a 50 ml centrifuge tube. Spiking solution was added and then 10 ml of acetonitrile with 0.1% of formic acid was added to reach the final volume of 10 ml. The tube was closed and shaken vigorously by hand at room temperature for 1 min to 3 min. Then the buffer-salt mixture (Quechers) was added and samples were shaken vigorously for 5 min using a shaker and centrifuged for 5 min at 5500 rpm. After this time 0.5 ml of sample and 10 µl of nicarbazin were transferred into the Eppendorf tube. Samples were diluted

to the final volume of 1 ml by water. Prepared samples were filtered with 0.22 µm PTFE into the injection vial for LC-MS/MS.

## Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SAN-TE/2020/12830 rev.1

## Conditions of the chemical determinations

### Reagents and chemicals

- Methanol, VWR Chemicals, catalogue no: 106035.2500, batch no: J1177935
- Ultrapure water
- Acetonitrile, VWR Chemicals, catalogue no: 83640.320, batch no: 21I072125,
- Formic acid, VWR Chemicals, catalogue no: 20318,297, batch no: 21J144020,
- QuEChERS Extraction Packet: (4 g MgSO<sub>4</sub> , 1 g NaCl, 1 g C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> i 0.5 g HOC(COOH)(CH<sub>2</sub>COONa)<sub>2</sub> x 1.5 H<sub>2</sub>O), Agilent Technologies part no: 5982-7650, serial no: 6669027-01,
- fludioxonil 99,82% Dr Ehrenstorfer/LGC, CAS No. 131341-86-1, batch no: G140218
- CGA192155 99,58% HPC Standards CAS No.126120-85-2, batch no: 810812
- nicarbazin, dr Ehrenstorfer, CAS No. 330-95-0 , batch no: G1050460

### Equipment

#### **Laboratory equipment:**

- Laboratory mill (knife mill), laboratory barcode: 33000000559
- Separator / MPW-352R, Fabric number: W352R051918, Laboratory barcode: 33000000277
- Shaker dedicated to QuEChERS – tube 50 ml / Tube Shaker Multi Reax, Serial number: 200157097, Laboratory barcode: 33000000088
- Shaker Sorvall Legend Mirco 17R Centrifuge ThermoScientific, Serial number: 42386120, Laboratory barcode: 33000000268
- Injection vials for LC-MS/MS – 2 ml
- Syringes filters, 0.22 µm pore size
- Syringes: 10 ml disposable syringes
- 50 ml centrifuge tubes with screw caps
- Eppendorf tubes: 1.5 ml
- Automatic pipettes: 0,5 -10 µl, 1-100 µl, 20-200 µl, 100-1000 µl, 1-10 ml
- Sample weight: analytical balance - Radwag PS 1000.X2, Serial number: 596082, Laboratory barcode 33000000053
- Weight of analytical standards: analytical balance with accuracy 0,00001 g. / Radwag XA.52.4Y.A.I, Serial number : 596088, nr. Laboratory barcode 33000000087
- Class A Measuring cylinder, 500 ± 2,5 ml for preparing mobile phase, marked with the DPL inscription
- Glass bottles 100 and 20 ml
- Freezer for analytical standard DPL1 33000000431
- Agilent Technologies LC-MS Triple Quad 6470 liquid chromatograph with Mass Hunter computer software.

#### **LC-MS/MS equipment:**

- 1260 Infinity II Binary Pump G7112B Serial No. DEAGO06796
- 1260 Infinity II Vialsampler G7129A Serial No. SEAEQ50974

- 1260 Infinity II Multicolumn Thermostat G7116A Serial No. DEAEM10470
- Solaris XE Serial No. 722020265
- Oil-free spiral compressor AVF 60 GOLD Serial No. IT 1085187
- 6470 LC/TQ G6470B Serial No. SG2209G202

#### Chromatographic parameters

Autosampler with cooling (constant temperature 10°C), injection volume 2 µl, injection mode – 200 µl/min. Chromatographic column: InfinityLab Poroshell 120 EC-C18 column with dimensions of 4.6x50 mm, and grain diameter 2.7 µm, series number USCFU40860 and guard column: InfinityLab Poroshell EC-C18 guard column with dimensions of 3.0 x 5 mm and grain diameter 2.7 µm, series number USCEC11811 maintaining a constant temperature of 35°C at the entrance and 35°C at the exit of the chromatographic column. Binary Pump: solvent A: 0.2% formic acid in the water, solvent B: 0.2% formic acid in methanol with LC-MS purity, flow rate 0.4 mL/min.

**Table A 1: Recovery results from method validation of Fludioxonil and its metabolite using the analytical method**

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Winter wheat (grain)	fludioxonil (247.0->180.0) - quantifier ion	0.01	94.9	4.3	-
		0.1	101.2	5.9	-
	fludioxonil (247.0->126.0) - qualifier ion	0.01	96.8	4.9	-
		0.1	102.1	4.6	-
Winter wheat (straw)	fludioxonil (247.0->180.0) - quantifier ion	0.01	105.3	2.9	-
		0.1	106.6	2.7	-
	fludioxonil (247.0->126.0) - qualifier ion	0.01	107.6	4.4	-
		0.1	107.0	1.6	-
Winter wheat (grain)	CGA192155 (201.0>65.1) - qualifier ion	0.01	72.8	3.3	-
		0.1	82.1	4.9	-
	CGA192155 (201.0 -> 91.1) - quantifier ion	0.01	79.4	5.6	-
		0.1	84.8	5.5	-
Winter wheat (straw)	CGA192155 (201.0>65.1) - qualifier ion	0.01	84.4	7.2	-
		0.1	92.4	9.1	-
	CGA192155 (201.0 -> 91.1) - quantifier ion	0.01	82.8	8.5	-
		0.1	89.9	3.4	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil and its metabolite residues in plant matrices**

	<b>Fludioxonil</b>																												
Specificity	<p>The applied LC-MS Triple Quad is a highly specific method due to the chromatographic separation and selective detection mass spectrometry-based system. Despite the high selectivity of detection in MRM mode, the identity of the tested analytes was confirmed.</p> <p>Confirmation of the presence of the analytes was obtained by comparing the signal ratios of the two MRM pairs. For the standards and samples tested, the relative signal response ratio for specific MRM transitions was determined using the MassHunter application, expressed as a percentage by the ratio of the qualifying ion response to the quantifier ion response.</p>																												
Calibration (type, number of data points)	<p>Calibration curves were performed on the winter wheat (grain, straw) matrix. 200 µl of winter wheat (grain, straw) matrix extracts, 700 µl of water, and 50 µl of ISTD were transferred to the Eppendorf tubes. Then 50 µl of appropriate solution of fludioxonil and CGA192155 standards and water were added. The final volumes of the prepared solution were always 1000 µl. In the next step, the Eppendorf tube was closed and shaken by hand. Prepared solutions were filtered with 0.22 µm PTFE into the injection vial for LC-MSMS.</p> <table><tr><th>Concentration of pre-prepared standard solution [µl/ml]</th><th>Matrix [µl]</th><th>Nicarbazin [µl]</th><th>fludioxonil + CGA192155 [µl]</th><th>Water [µl]</th></tr><tr><td>0.1</td><td rowspan="10">200</td><td rowspan="10">50</td><td rowspan="10">50</td><td>700</td></tr><tr><td>0.05</td><td>700</td></tr><tr><td>0.02</td><td>700</td></tr><tr><td>0.01</td><td>700</td></tr><tr><td>0.005</td><td>700</td></tr><tr><td>0.002</td><td>700</td></tr><tr><td>0.001</td><td>700</td></tr><tr><td>0.0005</td><td>700</td></tr><tr><td>0.0002</td><td>700</td></tr><tr><td>0.0001</td><td>700</td></tr></table>	Concentration of pre-prepared standard solution [µl/ml]	Matrix [µl]	Nicarbazin [µl]	fludioxonil + CGA192155 [µl]	Water [µl]	0.1	200	50	50	700	0.05	700	0.02	700	0.01	700	0.005	700	0.002	700	0.001	700	0.0005	700	0.0002	700	0.0001	700
Concentration of pre-prepared standard solution [µl/ml]	Matrix [µl]	Nicarbazin [µl]	fludioxonil + CGA192155 [µl]	Water [µl]																									
0.1	200	50	50	700																									
0.05				700																									
0.02				700																									
0.01				700																									
0.005				700																									
0.002				700																									
0.001				700																									
0.0005				700																									
0.0002				700																									
0.0001				700																									
Calibration range	<p>For monitoring purposes, the quantification was done in extracts from Winter wheat (grain, straw) matrices, respectively. The method showed to be linear up to 0.15 mg/kg for all analytes in the matrix with LC-MS/MS. A criterion for the acceptance of the linearity (<math>R^2 \geq 0.99</math>) is fulfilled for all analytes in the method.</p> <table><tr><th rowspan="2">Matrix</th><th rowspan="2">Linearity parameters</th><th colspan="2">Fludioxonil</th><th colspan="2">CGA192155</th></tr><tr><th>First transition (247.0-&gt;180.0 )</th><th>Second transition (247-&gt;126.0)</th><th>First transition (201.0 -&gt; 91.1)</th><th>Second transition (201,0&gt;65.1)</th></tr><tr><td rowspan="2">Winter wheat (grain)</td><td>Equation</td><td>y = 2.53 * x + 0.0017</td><td>y = 1.59 * x + 9.97E-004</td><td>y = 2.27 * x - 7.53E-004</td><td>y=0.43 * x - 8.274E-005</td></tr><tr><td>R<sup>2</sup></td><td>0.9973</td><td>0.9974</td><td>0.9944</td><td>0.9959</td></tr><tr><td>Winter wheat (straw)</td><td>Equation</td><td>y= 2.52 * x + 4.91E-004</td><td>y= 1.572843 * x + 6.60E-</td><td>y= 3.18 * x - 0.0012</td><td>y= 0.63 * x - 3.472E-005</td></tr></table>	Matrix	Linearity parameters	Fludioxonil		CGA192155		First transition (247.0->180.0 )	Second transition (247->126.0)	First transition (201.0 -> 91.1)	Second transition (201,0>65.1)	Winter wheat (grain)	Equation	y = 2.53 * x + 0.0017	y = 1.59 * x + 9.97E-004	y = 2.27 * x - 7.53E-004	y=0.43 * x - 8.274E-005	R <sup>2</sup>	0.9973	0.9974	0.9944	0.9959	Winter wheat (straw)	Equation	y= 2.52 * x + 4.91E-004	y= 1.572843 * x + 6.60E-	y= 3.18 * x - 0.0012	y= 0.63 * x - 3.472E-005	
Matrix	Linearity parameters			Fludioxonil		CGA192155																							
		First transition (247.0->180.0 )	Second transition (247->126.0)	First transition (201.0 -> 91.1)	Second transition (201,0>65.1)																								
Winter wheat (grain)	Equation	y = 2.53 * x + 0.0017	y = 1.59 * x + 9.97E-004	y = 2.27 * x - 7.53E-004	y=0.43 * x - 8.274E-005																								
	R <sup>2</sup>	0.9973	0.9974	0.9944	0.9959																								
Winter wheat (straw)	Equation	y= 2.52 * x + 4.91E-004	y= 1.572843 * x + 6.60E-	y= 3.18 * x - 0.0012	y= 0.63 * x - 3.472E-005																								



	Fludioxonil					
			004			
	R <sup>2</sup>	0.9988	0.9989	0.9947	0.9961	
Assessment of matrix effects is presented	yes					
Limit of determination/quantification	The limit of detection (LOD) is set at 0.0001 mg/kg. The Limit of Quantification (LOQ) was set at 0.01 mg/kg. Recovery tests were performed at this spiking level to verify the efficiency of the analytical method at LOQ.					

## Conclusion

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

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

No new or additional studies have been submitted.

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

#### A 2.1.2.3.1 Analytical method 1

##### A 2.1.2.3.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Anna Wróbel, 2022/ Synthos Agro

Report Validation included in the report: FLUDIO 025 GF Earthworm (*Eisenia andrei*) reproduction test, Anna Wróbel, MSc, 2022, Study code: G/23/21

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

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in artificial soil. The range of

linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev.

### Sample preparation for the chemical determinations

#### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the working solution at concentration 100 µg/mL in mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v). Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 <sup>2)</sup>	0.1	1	100 <sup>2)</sup>
100	0.2	1	20 <sup>1)</sup>
100	0.1	1	10 <sup>1)</sup>
100	0.05	1	5 <sup>1)</sup>
20	0.1	1	2 <sup>1)</sup>
10	0.1	1	1 <sup>1)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2 <sup>1)</sup>
1	0.1	1	0.1
0.5	0.1	1	0.05
0.1	0.1	1	0.01

1) Concentration level used for calibration.

2) Fortification solutions.

#### Preparation of Fortified Sample

For validation experiments, 10 g aliquot of untreated artificial soil were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Number of repetitions	Sample Weight [g]	Concentration Of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	2	10	--	--	0.0
Fortification (LOQ)	5	10	100	0.25	2.5
Fortification (10x LOQ)	5	10	1000	0.25	25.0

Sample of artificial soil an untreated (10 g) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 2.5 mg fludioxonil/kg and ten times higher of LoQ 25.0 mg fludioxonil/kg. This was done to ensure the result fits within the range of the respective standard curve.

#### Sample preparation for the chromatographic analysis

5 mL of mixture of acetonitrile for HPLC were added to 10 g of an artificial soil sample, shaken for 2 minute, and sonicated for 10 minutes. The sample was centrifuged and filtered through filter paper. The extraction was repeated with 5 mL of acetonitrile for HPLC. The eluate was diluted in ratio 1 – 1 with mixture of acetonitrile and 0.05% ortho-phosphoric acid (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

## Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SA TE/2020/12830 rev.1

## Conditions of the chemical determinations

### Chemicals:

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Acetonitrile	HPLC	POCH	1171/08/21	08.2024
			1078/09/21	09.2024
Ortho-phosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023

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

### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solution of fludioxonil at concentration 100.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v),
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v).

### Equipment:

Equipment	Size, Description	Manufacturer/Supplier
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pipettes	Various volumes	Eppendorf AG (Germany)
Balance	WPS 510/C	ZMP RADWAG (Poland)
Ultrasonic cleaner	Sonic-5	POLSONIC (Poland)
Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Chromatograph	Prominence- <i>i</i>	Shimadzu Corp. (Japan)
Laboratory	MVP-351e	MPW MED. INSTRUMENTS

centrifuge		(Poland)
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The following liquid chromatography parameters were used

Chromatographic System	Parameter
Chromatograph	High Performance Liquid Chromatography (HPLC)
Analytical Column	Shimadzu, Prominence-i (Shimadzu Corporation Japan)
Oven temperature	Kinetex 5µm C18 100Å, l = 150 mm, φ = 4,6 mm
Injection Volume	35°C
Mobile Phase	10 µL
Flow Rate	acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)
Wave length	0.83 mL/min
Detection System	215 nm
	Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Artificial soil	Fludioxonil	2.5	96.7	1.2	-
		25.0	94.9	0.8	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in artificial soil**

	Fludioxonil								
Specificity	The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the control matrix sample.								
Calibration (type, number of data points)	Working solutions of fludioxonil at the concentrations of 0.2, 0.5, 1, 2, 5, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.								
Calibration range	<div>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</div> <table><tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td>Fludioxonil</td><td>66343.3</td><td>-473.158</td><td>0.9999766</td></tr></table>	Analyte	Slope	Intercept	Coefficient	Fludioxonil	66343.3	-473.158	0.9999766
Analyte	Slope	Intercept	Coefficient						
Fludioxonil	66343.3	-473.158	0.9999766						
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	Limit of Quantification was estimated as the lowest								

	Fludioxonil
	<p>concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably <math>\leq 20\%</math>).</p> <p>The LoQ is 2.5 mg fludioxonil/kg artificial soil and equivalent to the calibration level at concentration 1.25 <math>\mu\text{g}</math> fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.</p> <p>The LoD is 0.4 mg fludioxonil/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.2 <math>\mu\text{g}</math> fludioxonil mL.</p>

## Conclusion

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

### A 2.1.2.3.2 Analytical method 2

#### A 2.1.2.3.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Aneta Gierbuszewska,, 2022/ Synthos Agro  
Anna Wróbel, 2022/ Synthos Agro

Report Validation included in the report: FLUDIO 025 GF Collembolean (*Folsomia candida*) Reproduction Test, Aneta Gierbuszewska, MSc, 2022, Study code: G/24/21  
Validation included in the report: FLUDIO 025 GF Predatory mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) reproduction test in soil, Anna Wróbel, MSc, 2022, Study code: G/25/21

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

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in artificial soil. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

## Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the working solution at concentration 100 µg/mL in mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v). Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000 <sup>2)</sup>	0.1	1	100 <sup>2)</sup>
100	0.2	1	20 <sup>1)</sup>
100	0.1	1	10 <sup>1)</sup>
100	0.05	1	5 <sup>1)</sup>
20	0.1	1	2 <sup>1)</sup>
10	0.1	1	1 <sup>1)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2 <sup>1)</sup>
1	0.1	1	0.1
0.5	0.1	1	0.05
0.1	0.1	1	0.01

1) Concentration level used for calibration.

2) Fortification solutions.

### Preparation of Fortified Sample

For validation experiments, 10 g aliquot of untreated artificial soil were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Number of repetitions	Sample Weight [g]	Concentration Of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	2	10	--	--	0.0
Fortification (LOQ)	5	10	100	0.25	2.5
Fortification (10x LOQ)	5	10	1000	0.25	25.0

Sample of artificial soil an untreated (10 g) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 2.5 mg fludioxonil/kg and ten times higher of LoQ 25.0 mg

fludioxonil/kg. This was done to ensure the result fits within the range of the respective standard curve.

#### Sample preparation for the chromatographic analysis

5 mL of mixture of acetonitrile for HPLC were added to 10 g of an artificial soil sample, shaken for 2 minute, and sonicated for 10 minutes. The sample was centrifuged and filtered through filter paper. The extraction was repeated with 5 mL of acetonitrile for HPLC. The eluate was diluted in ratio 1 – 1 with mixture of acetonitrile and 0.05% ortho-phosphoric acid (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

### **Results and discussions**

Confirmatory method not required due to specific method to the analytes. According to SAN-TE/2020/12830 rev.1

### **Conditions of the chemical determinations**

#### Chemicals:

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before Analysis	
Acetonitrile	HPLC	POCH	1171/08/21	08.2024
			1078/09/21	09.2024
Ortho-phosphoric acid	85% HPLC	SUPELCO	Z0721828108	31.07.2023

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

#### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solution of fludioxonil at concentration 100.0 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v),
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 µg/mL in mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid solution (50:50, v/v).

#### Equipment:

Equipment	Size, Description	Manufacturer/Supplier
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pippets	Various volumes	Eppendorf AG (Germany)
Balance	WPS 510/C	ZMP RADWAG (Poland)
Ultrasonic cleaner	Sonic-5	POLSONIC (Poland)
Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Chromatograph	Prominence- <i>i</i>	Shimadzu Corp. (Japan)
Laboratory centrifuge	MVP-351e	MPW MED. INSTRUMENTS (Poland)

The following liquid chromatography parameters were used

#### **Parameter**

### Chromatographic System

Chromatograph

Analytical Column

Oven temperature

Injection Volume

Mobile Phase

Flow Rate

Wave length

Detection System

High Performance Liquid Chromatography (HPLC)

Shimadzu, Prominence-i (Shimadzu Corporation Japan)

Kinetex 5µm C18 100Å, l = 150 mm, φ = 4,6 mm

35°C

10 µL

acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)

0.83 mL/min

215 nm

Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Artificial soil	Fludioxonil	2.5	107.7	0.4	-
		25.0	99.8	1.6	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in artificial soil**

	<b>Fludioxonil</b>								
Specificity	The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the control matrix sample.								
Calibration (type, number of data points)	Working solutions of fludioxonil at the concentrations of 0.2, 0.5, 1, 2, 5, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.								
Calibration range	<div>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</div> <table><tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td>Fludioxonil</td><td>66343.3</td><td>-473.158</td><td>0.9999766</td></tr></table>	Analyte	Slope	Intercept	Coefficient	Fludioxonil	66343.3	-473.158	0.9999766
Analyte	Slope	Intercept	Coefficient						
Fludioxonil	66343.3	-473.158	0.9999766						
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	<div>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably <math>\leq 20\%</math>).</div> <div>The LoQ is 2.5 mg fludioxonil/kg artificial soil and</div>								



	Fludioxonil
	<p>equivalent to the calibration level at concentration 1.25 µg fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.</p> <p>The LoD is 0.4 mg fludioxonil/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.2 µg fludioxonil/ mL.</p>

## Conclusion

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

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

### A 2.1.2.4.1 Analytical method 1

#### A 2.1.2.4.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Grażyna Hodorek, 2022/ Synthos Agro

Report Validation included in the report: FLUDIO 025 GF Daphnia magna, Acute Immobilisation Test, Grażyna Hodorek, MSc, 2022, Study code: W/31/21

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

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in Elendt M7 medium. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC - high performance liquid chromatography) with DAD (diode-array detection) detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

## Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the stock solution at concentration 1 mg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000	0.1	1	100
100	0.2	1	20
100	0.1	1	10 <sup>2)</sup>
100	0.05	1	5
20	0.1	1	2
10	0.1	1	1 <sup>1,2)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2
1	0.1	1	0.1 <sup>1)</sup>
0.5	0.1	1	0.05 <sup>1)</sup>
0.1	0.1	1	0.01 <sup>1)</sup>

1) Concentration level used for calibration.

2) Fortification solutions.

### Preparation of Fortified Sample

For validation experiments, 1 mL aliquot of untreated Elendt M7 medium were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Sample Volume [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	1	--	--	0.00
Fortification (LOQ)	1	1	0.05	0.05
Fortification (10x LOQ)	1	10	0.05	0.5

Sample of Elendt M7 medium an untreated (1 mL) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 0.05 mg fludioxonil/L and ten times higher of LoQ 0.5 mg fludioxonil/L. This was done to ensure the result fits within the range of the respective standard curve.

### Sample preparation for the chromatographic analysis

The Elendt M7 medium sample was taken and diluted with mixture acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50; v/v) in ratio 1 : 1. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

## Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SA TE/2020/12830 rev.1

## Conditions of the chemical determinations

### Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before analysis	
Acetonitrile	HPLC	POCH	1078/09/21	09.2024
Ortho-phosphoric acid	85% pure p.a.	SUPELCO	Z0721828108	31.07.2023

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

### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20 and 100 µg/mL in mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v).

### Equipment:

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pippets	Various volumes	Eppendorf AG (Germany)
Chromatograph	Prominence- <i>i</i>	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

### Chromatographic System

Chromatograph

Analytical Column

Oven temperature

Injection Volume

Mobile Phase

Flow Rate

Wave length

### Detection System

### Parameter

High Performance Liquid Chromatography (HPLC)

Shimadzu, Prominence-*i* (Shimadzu Corporation Japan)

Kinetex 5µm C18 100Å, l = 150 mm, □ = 4,6 mm

35°C

20 µL

acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)

0.83 mL/min

215 nm

Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Elendt M7 medium	Fludioxonil	0.05	101.8	1.8	-
		0.5	100.8	0.6	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in Elendt M7 medium**

	Fludioxonil								
Specificity	The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.								
Calibration (type, number of data points)	<p>Working solutions of fludioxonil at the concentrations of 0.01, 0.05, 0.1, 0.50 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</p> <p>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</p> <table><tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td>Fludioxonil</td><td>132406</td><td>-171.205</td><td>0.9997110</td></tr></table>	Analyte	Slope	Intercept	Coefficient	Fludioxonil	132406	-171.205	0.9997110
Analyte	Slope	Intercept	Coefficient						
Fludioxonil	132406	-171.205	0.9997110						
Calibration range	The range of linearity of the analytical graph is from 0.01 µg/mL to 1 µg/mL. The range of calibration curve of fludioxonil is equivalent to range from 0.02 mg fludioxonil/L to 2 mg fludioxonil/L in Elendt M7 medium.								
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	<p>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably <math>\leq 20\%</math>).</p> <p>The LoQ is 0.05 mg fludioxonil/L Elendt M7 medium and equivalent to the calibration level at concentration 0.025 µg fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.</p> <p>The LoD is 0.02 mg fludioxonil/L Elendt M7 medium and equivalent to the lowest calibration standard i.e. 0.01 µg</p>								

	<b>Fludioxonil</b>
	fludioxonil/mL.

## Conclusion

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


### A 2.1.2.4.2 Analytical method 2

#### A 2.1.2.4.2.1 Method validation

Comments of zRMS:	Method is accepted
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Validation included in the following reports:

Reference:

, 2022/ Synthos Agro  
, 2022/ Synthos Agro

Report

Validation included in the report: FLUDIO 025 GF *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test, Grażyna Hodorek, MSc, 2022, Study code: W/32/21  
Validation included in the report: FLUDIO 025 Rainbow trout, Acute Toxicity Testing, Ewa Nierzędska, MSc, 2022, Study code: W/33/21

Guideline(s):

SANTE/2020/12830 rev.1

Deviations:

No

GLP:

Yes

Acceptability:

Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in water. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

## Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with

mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the stock solution at concentration 1 mg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Fina Concentration [µg/mL]
1000	0.1	1	100
100	0.2	1	20
100	0.1	1	10 <sup>2)</sup>
100	0.05	1	5
20	0.1	1	2
10	0.1	1	1 <sup>1,2)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2
1	0.1	1	0.1 <sup>1)</sup>
0.5	0.1	1	0.05 <sup>1)</sup>
0.1	0.1	1	0.01 <sup>1)</sup>

1) Concentration level used for calibration.

2) Fortification solutions.

#### Preparation of Fortified Sample

For validation experiments, 1 mL aliquot of untreated ElenDt M7 medium were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Sample Volume [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	1	--	--	0.00
Fortification (LOQ)	1	0.5	0.1	0.0005
Fortification (10x LOQ)	1	5.0	0.1	0.005

Sample of water an untreated (100 mL) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 0.0005 mg fludioxonil/L and ten times higher of LoQ 0.005 mg fludioxonil/L. This was done to ensure the result fits within the range of the respective standard curve.

#### Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of methanol, twice with 5 mL of deionised water. Following the sample introduction the column was dried under vacuum for 5 minutes. The active substance was eluted with twice with 5 mL of methanol. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was redissolved in mixture acetonitrile and 0.05% ortho-phosphoric acid (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

#### **Results and discussions**

Confirmatory method not required due to specific method to the analytes. According to SAN-TE/2020/12830 rev.1

#### **Conditions of the chemical determinations**

### Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before analysis	
Acetonitrile	HPLC	POCH	1171/08/21 08.2024	1171/08/21 08.2024
			1078/09/21 09.2024	1078/09/21 09.2024
Methanol	pure	POCH	1158/11/20	11.2025
Ortho-phosphoric acid	85% pure p.a.	SUPELCO	Z0721828108	31.07.2023
SUPELLEAN ENVI-18 SPE	3 mL, 500 mg	Supelco	12546401	12.05.2026

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

### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20 and 100 µg/mL in mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v).

### Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pippets	Various volumes	Eppendorf AG (Germany)
Rotary vacuum evaporator with water bath	RV 05 basic HB 4 basic	IKA - WERKE (Germany)
SPE vacuum manifold	Visiprep	Supelco (USA)
SPE cartridges	Supelclean ENVI-18	Supelco (USA)
Chromatograph	Prominence- <i>i</i>	Shimadzu Corp. (Japan)
Rotary vacuum evaporator with water bath	RV 10 digital HB 10 digital	IKA - WERKE (Germany)

The following liquid chromatography parameters were used

#### **Chromatographic System**

Chromatograph

Analytical Column

Oven temperature

Injection Volume

Mobile Phase

Flow Rate

Wave length

#### **Detection System**

#### **Parameter**

High Performance Liquid Chromatography (HPLC)

Shimadzu, Prominence-*i* (Shimadzu Corporation Japan)

Kinetex 5µm C18 100Å, l = 150 mm, φ = 4,6 mm

35°C

20 µL

acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)

0.83 mL/min

215 nm

Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water	Fludioxonil	0.0005	108.0	1.3	-
		0.005	104.8	2.7	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in water**

	<b>Fludioxonil</b>								
Specificity	The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.								
Calibration (type, number of data points)	<p>Working solutions of fludioxonil at the concentrations of 0.01, 0.05, 0.1, 0.50 and 1.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</p> <p>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</p> <table><tr><td><b>Analyte</b></td><td><b>Slope</b></td><td><b>Intercept</b></td><td><b>Coefficient</b></td></tr><tr><td>Fludioxonil</td><td>132406</td><td>-171.205</td><td>0.9997110</td></tr></table>	<b>Analyte</b>	<b>Slope</b>	<b>Intercept</b>	<b>Coefficient</b>	Fludioxonil	132406	-171.205	0.9997110
<b>Analyte</b>	<b>Slope</b>	<b>Intercept</b>	<b>Coefficient</b>						
Fludioxonil	132406	-171.205	0.9997110						
Calibration range	The range of linearity of the analytical graph is from 0.01 µg/mL to 1 µg/mL. The range of calibration curve of fludioxonil is equivalent to range from 0.0001 mg fludioxonil/L to 0.01 mg fludioxonil/L in water.								
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	<p>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).</p> <p>The LoQ is 0.0005 mg fludioxonil/L water and equivalent to the calibration level at concentration 0.05 µg fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.</p> <p>The LoD is 0.0001 mg fludioxonil/L water and equivalent to the lowest calibration standard i.e. 0.01 µg fludioxonil/mL.</p>								



## Conclusion

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

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

No new or additional studies have been submitted

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

No new or additional studies have been submitted

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

#### A 2.1.2.7.1 Analytical method 1

##### A 2.1.2.7.1.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Elżbieta Kulec-Płoszczyca, MSc, 2022/ Synthos Agro  
Elżbieta Kulec-Płoszczyca, MSc, 2022/ Synthos Agro

Report Validation included in the report: FLUDIO 025 GF Bumblebees (*Bombus spp.*), Acute Oral Toxicity Test, Elżbieta Kulec-Płoszczyca, MSc, 2022, Study code: B/67/21  
Validation included in the report: FLUDIO 025 GF Honeybees (*Apis mellifera L.*), Chronic Oral Toxicity Test, Elżbieta Kulec-Płoszczyca, MSc, 2022, Study code: B/02/22

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

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in sucrose solution. The range of linearity of the analytical graphs, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

## Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the stock solution at concentration 1 mg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000	0.1	1	100 <sup>2)</sup>
100	0.2	1	20
100	0.1	1	10
100	0.05	1	5
20	0.1	1	2
10	0.1	1	1 <sup>1)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2
1	0.1	1	0.1 <sup>1)</sup>
0.5	0.1	1	0.05 <sup>1)</sup>
0.1	0.1	1	0.01 <sup>1)</sup>

1) Concentration level used for calibration.

2) Fortification solutions.

### Preparation of Fortified Sample

For validation experiments, 1 g aliquot of untreated sucrose solution were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Sample Volume [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	1	--	--	0.00
Fortification (LOQ)	1	5	0.1	0.5
Fortification (10x LOQ)	1	100	0.05	5.0

Sample of water an untreated (1 g) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 0.5 mg fludioxonil/kg and ten times higher of LoQ 5.0 mg fludioxonil/kg. This was done to ensure the result fits within the range of the respective standard curve.

### Sample preparation for the chromatographic analysis

First, 1 g sucrose sample was weighted into a volumetric flask with a capacity of 10 mL and added 4 mL of 0.05% ortho-phosphoric acid (v/v). Next, the volume was made up to 10 mL with acetonitrile for HPLC.

The eluate was diluted with mixture acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

## Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SAN-TE/2020/12830 rev.1.

## Conditions of the chemical determinations

### Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before analysis	
Acetonitrile	HPLC	POCH	1078/09/21	09.2024
Ortho-phosphoric acid	85% pure p.a.	SUPELCO	Z0721828108	31.07.2023

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

### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20 and 100 µg/mL in mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v).

### Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Technical Balance	WPS/510C	Radwag (Poland)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pippets	Various volumes	Eppendorf AG (Germany)
Chromatograph	Prominence-i	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

### Chromatographic System

Chromatograph

Analytical Column

Oven temperature

Injection Volume

Mobile Phase

### Parameter

High Performance Liquid Chromatography (HPLC)

Shimadzu, Prominence-i (Shimadzu Corporation Japan)

Kinetex 5µm C18 100Å, l = 150 mm, □ = 4,6 mm  
35°C

20 µL

acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)

Flow Rate	0.83 mL/min
Wave length	215 nm
Detection System	Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Sucrose solution	Fludioxonil	0.05	105.0	3.0	-
		0.5	99.6	0.4	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in Sucrose solution**

	<b>Fludioxonil</b>								
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.								
Calibration (type, number of data points)	<p>Working solutions of fludioxonil at the concentrations of 0.01, 0.05, 0.1, 0.5 and 1 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</p> <p>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</p> <table><tr><td><b>Analyte</b></td><td><b>Slope</b></td><td><b>Intercept</b></td><td><b>Coefficient</b></td></tr><tr><td>Fludioxonil</td><td>132406</td><td>-171.205</td><td>0.9997110</td></tr></table>	<b>Analyte</b>	<b>Slope</b>	<b>Intercept</b>	<b>Coefficient</b>	Fludioxonil	132406	-171.205	0.9997110
<b>Analyte</b>	<b>Slope</b>	<b>Intercept</b>	<b>Coefficient</b>						
Fludioxonil	132406	-171.205	0.9997110						
Calibration range	The range of linearity of the analytical graph is from 0.01 µg/mL to 1 µg/mL. The range of calibration curve of fludioxonil is equivalent to range from 0.1 mg fludioxonil/kg to 10 mg fludioxonil/kg in sucrose solution.								
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	<p>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).</p> <p>The LoQ is 0.5 mg fludioxonil/kg sucrose solution and equivalent to the calibration level at concentration 0.05 µg fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest</p>								

	Fludioxonil
	detectable concentration or amount of an analyte in a sample.  The LoD is 0.1 mg fludioxonil/kg sucrose solution and equivalent to the lowest calibration standard i.e. 0.01 µg fludioxonil/mL.

## Conclusion

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

### A 2.1.2.7.2 Analytical method 2

#### A 2.1.2.7.2.1 Method validation

Comments of zRMS:	Method is accepted
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Reference: Validation included in the following reports:

Elżbieta Kulec-Płoszczyca, MSc, 2022/ Synthos Agro  
Elżbieta Kulec-Płoszczyca, MSc, 2022/ Synthos Agro

Report Validation included in the report: FLUDIO 025 GF Bumblebees (*Bombus spp.*), Acute Contact Toxicity Test, Elżbieta Kulec-Płoszczyca, MSc, 2022, Study code: B/68/21  
Validation included in the report: FLUDIO 025 GF Honeybees (*Apis mellifera L.*), Larval Toxicity Test, Repeated Exposure, Elżbieta Kulec-Płoszczyca, MSc, 2022, Study code: B/01/22

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

Deviations: No

GLP: Yes

Acceptability: Yes

## Materials and methods

The analytical method was developed for the determination of fludioxonil in water. The range of linearity of the analytical graphs, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, stability stock solution, limit of quantification and detection were determined.

The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

## Sample preparation for the chemical determinations

### Stock and standard solutions

The stock solution of fludioxonil with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of fludioxonil into a volumetric flasks with a capacity of 10 mL, dissolving in acetonitrile for HPLC, and next the volume was made up to 10 mL with the same solvent. The working solutions of fludioxonil with a concentration of 100 µg/mL were prepared by dilution of the stock solution with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v). Calibration and fortification solutions containing of fludioxonil were prepared by dilution of the stock solution at concentration 1 mg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50, v/v) as exemplarily described in the table below:

Take solution at concentration [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]
1000	0.1	1	100 <sup>2)</sup>
100	0.2	1	20 <sup>1)</sup>
100	0.1	1	10 <sup>1)</sup>
100	0.05	1	5 <sup>1)</sup>
20	0.1	1	2 <sup>1)</sup>
10	0.1	1	1 <sup>1)</sup>
5	0.1	1	0.5 <sup>1)</sup>
2	0.1	1	0.2 <sup>1)</sup>
1	0.1	1	0.1
0.5	0.1	1	0.05
0.1	0.1	1	0.01

1) Concentration level used for calibration.

2) Fortification solutions.

#### Preparation of Fortified Sample

For validation experiments, 1 g aliquot of untreated sucrose solution were spiked with appropriate volumes of common fortification solutions of fludioxonil. The following fortification scheme was used:

Sample Type	Sample Volume [g]	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	1	--	--	0.00
Fortification (LOQ)	1	10	0.1	1.0
Fortification (10x LOQ)	1	100	0.1	10.0

Sample of water an untreated (1 mL) was spiked with the solution of fludioxonil to achieve fortification levels at the limit of quantification i.e. 1.0 mg fludioxonil/L and ten times higher of LoQ 10.0 mg fludioxonil/L. This was done to ensure the result fits within the range of the respective standard curve.

#### Sample preparation for the chromatographic analysis

The water sample was taken and diluted with mixture acetonitrile for HPLC and 0.05% ortho-phosphoric acid (50:50; v/v) in ratio 1 : 1. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

## Results and discussions

Confirmatory method not required due to specific method to the analytes. According to SAN-TE/2020/12830 rev.1.

## Conditions of the chemical determinations

### Chemicals

Chemical	Grade	Manufacturer/ Supplier	Batch Number	Expiry date
Deionized water	HPLC	Łukasiewicz-IPO*	Fresh prepared before analysis	
Acetonitrile	HPLC	POCH	1078/09/21	09.2024
Ortho-phosphoric acid	85% pure p.a.	SUPELCO	Z0721828108	31.07.2023

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

### Reagents and solvents

- 0.05% ortho-phosphoric acid solution in deionized water (v/v),
- mixture of acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50, v/v),
- standard solution of 1 mg/mL of fludioxonil in acetonitrile for HPLC,
- working solutions of fludioxonil at concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20 and 100 µg/mL in mixture of acetonitrile for HPLC and 0.05% orthophosphoric acid (50:50, v/v).

### Equipment

Equipment	Size, Description	Manufacturer/Supplier
Analytical Balance	Adventurer Pro AV 114CM	Ohaus Corporation (USA)
Volumetric flasks	Various volumes	Glassco (Germany)
Variable volume single-channel pippets	Various volumes	Eppendorf AG (Germany)
Chromatograph	Prominence- <i>i</i>	Shimadzu Corp. (Japan)

The following liquid chromatography parameters were used

### Chromatographic System

Chromatograph

Analytical Column

Oven temperature

Injection Volume

Mobile Phase

Flow Rate

Wave length

### Detection System

### Parameter

High Performance Liquid Chromatography (HPLC)

Shimadzu, Prominence-*i* (Shimadzu Corporation Japan)

Kinetex 5µm C18 100Å, l = 150 mm, φ = 4,6 mm

35°C

10 µL

acetonitrile for HPLC : 0.05% ortho-phosphoric acid (50 : 50, v/v)

0.83 mL/min

215 nm

Diode Array Detector

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

Matrix	Analyte	Fortification level (mg/kg) (n = x)	Mean recovery (%)	RSD (%)	Comments
Water	Fludioxonil	1	105.0	1.0	-
		10	99.4	0.9	-

**Table A 2: Characteristics for the analytical method used for validation of Fludioxonil residues in water**

	Fludioxonil								
Specificity	The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control matrix, and fortified samples of matrix. Considering the results of the analysis, no signal of detected substance were overlapping with matrix signal of the control samples in the experimental conditions. Therefore, the specificity of the method was demonstrated. Furthermore, any interference is directly apparent and would be observed in the chromatogram of the matrix control sample.								
Calibration (type, number of data points)	<p>Working solutions of fludioxonil at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded.</p> <p>The equations of the calibration line were presented as the linear equation; <math>y = ax + b</math> (a – slope, b - intercept). The linear coefficient <math>r^2</math> must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.</p> <table><tr><th>Analyte</th><th>Slope</th><th>Intercept</th><th>Coefficient</th></tr><tr><td>Fludioxonil</td><td>66343.3</td><td>-473.158</td><td>0.9999766</td></tr></table>	Analyte	Slope	Intercept	Coefficient	Fludioxonil	66343.3	-473.158	0.9999766
Analyte	Slope	Intercept	Coefficient						
Fludioxonil	66343.3	-473.158	0.9999766						
Calibration range	The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of fludioxonil is equivalent to range from 0.4 mg fludioxonil/L to 40 mg fludioxonil/L in water.								
Assessment of matrix effects is presented	yes								
Limit of determination/quantification	<p>Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).</p> <p>The LoQ is 1.0 mg fludioxonil/L water and equivalent to the calibration level at concentration 0.5 µg fludioxonil/mL.</p> <p>The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.</p> <p>The LoD is 0.4 mg fludioxonil/L water and equivalent to the lowest calibration standard i.e. 0.2 µg fludioxonil/mL.</p>								



## **Conclusion**

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