FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: Diflufenikan 500 SC

Product name(s): -

Chemical active substance:

diflufenican, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: Pestila Sp. z o.o. / ProAgri International Sp. z o.o.
Submission date: January 2023

MS Finalisation date: September 2023**,** January 2024, April 2024

Version history

When	What
September 2023	ZRMS assessment of dRR
January 2024	The final Registration Report
April 2024	ZRMs made correction in line to MRiRW comments.

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5 Analytical methods

5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are available for the active substance and relevant impurities in the plant protection product.
Noticed data gaps:
- none

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.
Noticed data gaps (minor) are:
- ILV method for drinking water,
- methods for the analysis of body fluids and tissues.

These data gaps can be covered after authorisation within 2 years.

Commodity/crop	Supported/ Not supported
Winter wheat, triticale	Supported
Winter barley	Supported
Rye	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 diflufenican in plant protection product is provided as follows:

Comments of evaluator:	The proposed analytical method is suitable for the determination of the active substance diflufenican in the plant protection product coded Diflufenikan 500 SC. The proposed analytical method has been fully validated in terms of interference, specificity, linearity, recovery and precision. The proposed method fulfils the requirements of SANCO/3030/99 rev.5 guidance. The validation of the analytical method has been accepted

Reference:	5.1.1/01 5.1.1/02
Report	DIFLUFENIKAN 500 SC. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage. Kupiec J., 2022, report no. BF – 24/22
Guideline(s):	Yes, SANCO/3030/99 rev.5 (22/03/19)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Determination of diflufenican in Diflufenikan 500 SC was performed with high performance liquid chromatography (HPLC) with a UV-VIS DAD detector and external standard.

Equipment and chromatographic conditions for prothioconazole analysis

- Shimadzu HPLC DAD UV-VIS
- Column: Kinetex ~~Biphenyl C18~~ EVO, 250 x 4.6 mm, 5µm
- Analytical balance
- Volumetric flasks
- Volumetric pipette
- Autosampler vials
- Ultrasonic bath, POLSONIC
- Syringe filters PTFE, 0.22 µm
- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC-Super Gradient, POCh
- Orthophosphoric acid 85%, Chempur
- Diflufenican - analytical standard
- Oven temperature: ~~35~~ 30 ºC
- Flow rate: 1.0 mL/min
- Wavelength λ = ~~192~~ 206 nm
- Volume injection: 5 µL
- Mobile phase composition: CH3CN + H3PO4 0.1 % aq. ~~(55 + 45)~~ (60+40) v/v

Under these chromatographic conditions, the retention time was ~~~11.3 ± 0.1~~ 13.0 ± 0.3 min. The total analysis time was ~~20~~ 25 min.

The preparation of standard solution

50.39 mg of Diflufenican standard was weighed (with the accuracy of 0.01 mg) into the 10 ml volumetric flask and acetonitrile was added to the nominal volume. Solution was diluted and analyzed.

The preparation of specimen solutions

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

The preparation of placebo solution

125.62 mg of placebo was weighed (with the accuracy of 0.01 mg) into the 25 ml volumetric flask. 5 ml water and acetonitrile was added, stirred and the flask was put into the ultrasonic bath for 5 min. After cooling acetonitrile was added to the nominal volume, solutions of examined specimen were passed through syringe filters. Solutions were diluted and analyzed.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances prothioconazole in plant protection product Diflufenikan 500 SC

	Diflufenican
Author(s), year	Kupiec J., 2022
Principle of method	SANCO/3030/99 rev.5, 22 March 2019
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of the analytical method was assessed using five diflufenican standard solutions in the concentration range from 0.3510 mg/mL to 0.7019 mg/mL (70 – 140% of declared content). y = 22 857 300.8703*x + 510 311.7622 Correlation coefficient: $R^2 = 0.999$ Required: $R^2 \geq 0.99$
Precision – Repeatability Mean n = 6 (%RSD)	Hr = ~~0.13~~ 0.08 Required: Hr ≤ 1 RSD = 0.12 Required: RSD ≤ 1.53
Accuracy n = 12 (% Recovery)	Total recovery: 100% (range: 97.9% - 102.9%) Required: 97% - 103%
Interference/ Specificity	Fulfilled.
Comment	No comments.

Conclusion.

The HPLC method with a UV-VIS DAD detector, used to quantify diflufenican in Diflufenikan 500 SC was fully validated. Method validation included linearity, non-analyte interference, precision, ~~accuracy~~ recovery and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

Not relevant. The product Diflufenikan 500 SC does not contain relevant impurities.

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

Not relevant. The product Diflufenikan 500 SC does not contain materials of toxicological, ecotoxicological or environmental concern.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For diflufenican suspension concentrate CIPAC Method 462/TC/M/3 (CIPAC Handbook H, p.148) is suitable.

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 diflufenican for the generation of pre-authorization data is given in the following table. The detailed evaluation of 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: diflufenican				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Sucrose solution (Bumbble bee-oral, acute) (Ecotoxicology)	Primary	10.0 mg/kg	HPLC with DAD detection	Kulec-Płoszczyca E., 2022 / Study code: B-100-22
1% solution of triton in water (Bumbble bee-contact, acute) (Ecotoxicology)	Primary	1.0 mg/L	HPLC with DAD detection	Kulec-Płoszczyca E., 2022 / Study code: B-102-22
Sediment and Smart&Barko medium (*Myriophyllum spicatum)* (Ecotoxicology)	Primary	0.05 mg/kg (sediment) 0.0005 mg/L (Smart&Barko Medium)	HPLC with DAD detection	Czarnecka M., 2022 / Study code: W-06-22
Elendt M7 medium (*Daphnia magna)* (Ecotoxicology)	Primary	0.05 mg/L	HPLC with DAD detection	Czarnecka M., 2022 / Study code: W-07-22
APP medium (*Raphidocelis subcapitata)* (Ecotoxicology)	Primary	0.01 mg/L	HPLC with MS/MS detection	Czarnecka M., 2022 / Study code: W-08-22
Water (*Lemna gibba)* (Ecotoxicology)	Primary	0.0005 mg/L	HPLC with DAD detection	Czarnecka, M., 2022 / Study code: W-09-22
Artificial soil (*Eisenia andrei)* (Ecotoxicology)	Primary	10.0 mg/kg	HPLC with DAD detection	Pieczka P., 2021 / Study code: G-89-21
Water (Vegetative Vigour; Seedling Emergence and Seedling Growth) (Ecotoxicology)	Primary	0.05 mg/L	HPLC with DAD detection	Pieczka P., 2021 / Study code: G-91-21
Water solution (Honeybees-larval, repeated) (Ecotoxicology)	Primary	0.0025 g/L	LC-MS/MS	Mautino G., 2023 / Study code: 1004.1H.SAG22, Test Site Code: 22354-02R
Sucrose solution	Primary	2.021 mg/kg	LC-MS/MS	Mautino G., 2023 / Study

Component of residue definition: diflufenican				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
(Honeybees-chronic, oral) (Ecotoxicology)				code: 1003.1H.SAG22, Test Site Code: 22354-01R

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 in the plant protection product are already submitted in accordance with the requirements set out in point 5.2.1.

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

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

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

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	diflufenican	0.01 mg/kg	Regulation (EU) No 2017/623 Annex II
Plant, high acid content		0.01 mg/kg	
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	
Plant, high oil content		0.01 mg/kg	
Muscle	diflufenican	0.02 mg/kg	Regulation (EU) No 2017/623 Annex II
Milk		0.01 mg/kg	
Eggs		0.02 mg/kg	
Fat		0.02 mg/kg	
Liver, kidney		0.02 mg/kg	
Soil (Ecotoxicology)	diflufenican	0.05 mg/kg	common limit
Drinking water (Human toxicology)	diflufenican	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	diflufenican	0.25 mg a.s./L (EC_{50} for *Scenedesmus subspicatus*)	EFSA Scientific Report (2007) 122, 1-84
Air	diflufenican	33 µg/m^3 (AOEL sys = 0.11 mg/kg bw/day)	Calculated according to SANTE/2020/12830, Rev.1 24. February 2021
Tissue (meat or liver)	diflufenican	not required	not classified as T / T+
Body fluids		not required	not classified as T / T+

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

An overview on the acceptable methods and possible data gaps for analysis of diflufenican in plant matrices is given in the following tables. No new studies have been submitted with this application.

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: diflufenican				
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	GC-ECD DFG S19	Bacher R. 2002g/DAR, 2006/Doc No C028188
	ILV	0.01 mg/kg	GC-ECD DFG S19	Thom M. 2003a/DAR. 2006/Doc. No C031483
	Confirmatory (if required)	-	-	-
High acid content	Primary	0.01 mg/kg	GC-ECD DFG S19	Bacher R. 2002g/DAR, 2006/Doc No C028188
	ILV	0.01 mg/kg	GC-ECD DFG S19	Thom M. 2003a/DAR. 2006/Doc. No C031483
	Confirmatory (if required)	-	-	-
High oil content	Primary	0.02 mg/kg	GC-ECD DFG S19	Bacher R. 2002g/DAR, 2006/Doc No C028188
	ILV	0.01 mg/kg	GC-ECD DFG S19	Thom M. 2003a/DAR. 2006/Doc. No C031483
	Confirmatory (if required)	-	-	-
High protein/high starch content (dry)	Primary	0.02 mg/kg 0.02 mg/kg	GC-ECD GC-ECD	Sharpe J.P. 1984b/DAR. 2006/Doc No R000944 Maycey P.A., Outram J.R. 1987i/DAR, 2006/ Doc No: R001011
	ILV	0.01 mg/kg	GC-ECD DFG S19	Klumpp M. 2001a/DAR. 2006/ Doc No: C018307
	Confirmatory (if required)	0.01 mg/kg	GC-ECD DFG S19	Class T. 2001b/DAR, 2006/Doc No: C013331

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	-
Not required, because:	Not provided during the EU review

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 diflufenican in animal matrices is given in the following tables. No new studies have been submitted with this application.

Table 5.3-4: 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: diflufenican				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Milk	Primary	0.01 mg/kg 0.01 mg/kg	AR 119-96 GC-MS DFG S19	Guillet M., Simonin B.1996a/ DAR, 2006/Doc No: R002767 Class T. 1999c/DAR, 2006/Doc. No R004321
	ILV	0.01 mg/kg	GC-MS DFG S19	Klumpp M. 2002a/DAR, 2006/ Doc No C022357
	Confirmatory (if required)	-	-	-
Eggs	Primary	0.02 mg/kg	AR 119-96	Guillet M., Simonin B.1996a/ DAR, 2006/Doc No: R002767
	ILV	0.02 mg/kg	GC-MS DFG S19	Klumpp M. 2002a/DAR, 2006/Doc No C022357
	Confirmatory (if required)	-	-	-
Muscle	Primary	0.02 mg/kg 0.02 mg/kg	AR 119-96 GC-MS DFG S19	Guillet M., Simonin B.1996a/ DAR, 2006/Doc No: R002767 Class T. 1999c/DAR, 2006/Doc. No R004321
	ILV	0.02 mg/kg	GC-MS DFG S19	Klumpp M. 2002a/DAR, 2006/ Doc No C022357
	Confirmatory (if required)	-	-	-
Kidney, liver	Primary	0.02 mg/kg	AR 119-96	Guillet M., Simonin B.1996a/ DAR, 2006/Doc No: R002767
	ILV	0.02 mg/kg	GC-MS DFG S19	Klumpp M. 2002a/DAR, 2006/ Doc No C022357
	Confirmatory (if required)	-	-	-

Table 5.3-5: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	-
Not required, because:	Not provided during the EU review. No residues are expected in foodstuff of animal origin.

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 diflufenican in soil is given in the following table. No new studies have been submitted with this application.

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

Component of residue definition: diflufenican			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.002 mg/kg	LC-MS/MS	Bacher R. 2002f/DAR, 2006/Doc No C025918
Confirmatory	0.002 mg/kg	GC-MS	Doran A.M., McGuire G.M. 2002a/DAR, 2006/Doc No. C025222

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 diflufenican in surface and drinking water is given in the following tables. No new studies have been submitted with this application.

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

Component of residue definition: diflufenican				
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	LC-MS/MS	Bacher R. 2002e/DAR, 2006/Doc No: C026100
	ILV	-	-	-
	Confirmatory	-	-	-
Surface water	Primary	0.05 µg/L	LC-MS/MS	Bacher R. 2002e/DAR, 2006/Doc No: C026100
	Confirmatory	-	-	-

Data gap:
- ILV method is required by the REGULATION (EU) No 284/2013 and SANTE/2020/12830, Rev.1

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 diflufenican in air is given in the following tables. No new studies have been submitted with this application.

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

Component of residue definition: diflufenican			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.4 µg/m^3	LC-MS/MS	Bacher R. 2002h/DAR, 2006/Doc No: C025825
Confirmatory	-	-	-

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

Methods for body fluids and tissues are not required, because diflufenican is not considered to be toxic or very toxic (T / T+) nor is it classified according to CLP regulatory as follows: Acute toxicity (cat. 1 - 3), CMR (cat. 1) or STOT (cat. 1).

Data gap:
- Methods are required by the REGULATION (EU) No 284/2013 and SANTE/2020/12830, Rev.1

5.3.2.8 Other studies/ information

Summary of validation of analytical methods used in dRR section 9 (Ecotoxicology) are provided in Appendix 2.

Appendix 1 Lists of data considered in support of the evaluation

Tables considered not relevant can be deleted as appropriate.

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

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
KCP 5.1.1/01 KCP 5.1.1/02	Kupiec J.	2022	DIFLUFENIKAN 500 SC. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage. Report No BF – 24/22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Published: no	N	Pestila* ProAgri**
KCP 5.1.2/01 filled as KCP 10.3.1.1.1/02	Kulec-Płoszczyca E.	2022	Diflufenikan 500 SC Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test Study code: B-100-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/02 filled as KCP 10.3.1.1.2/02	Kulec-Płoszczyca E.	2022	Diflufenikan 500 SC Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test Study code: B-102-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/03 (filed as KCP 10.2.1.3/02)	Czarnecka M.	2022	Diflufenikan 500 SC Water-sediment *Myriophyllum spicatum* toxicity test Study code: W-06-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**

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 5.1.2/04 (filed as KCP 10.2.1.3/01)	Czarnecka M.	2022	Diflufenikan 500 SC *Daphnia magna*, Acute Immobilisation Test Study code: W-07-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/05 (filed as KCP 10.2.1.3/01)	Czarnecka M.	2022	Diflufenikan 500 SC *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test Study code: W-08-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/06 (filed as KCP 10.2.1.4/01)	Czarnecka M.	2022	Diflufenikan 500 SC *Lemna gibba* CPCC 310, Growth inhibition test Study code: W-09-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/07 (filed as KCP 10.4.1.1/01)	Pieczka P.	2022	Diflufenikan 500 SC Earthworm reproduction test *(Eisenia andrei)* Study code: G-89-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/08 (filed as KCP 10.6.2/02)	Pieczka P.	2022	Diflufenikan 500 SC Terrestial Plant Test: Vegetative Vigour Test Study code: G-91-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/09	Mautino G.	2023	Analytical Phase Report Effects of DIFLUFENIKAN 500 SC (diflufenican 500 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure Study Code: 1004.1H.SAG22, Test Site Code: 22354-02R	N	Pestila* ProAgri**

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
			Renolab S.r.l. / SAGEA Centro di Saggio s.r.l. GLP Unpublished		
KCP 5.1.2/10	Mautino G.	2023	Analytical Phase Report Effects of DIFLUFENIKAN 500 SC (diflufenican 500 g/L) on Honeybees (*Apis mellifera* L.) in the laboratory – Chronic Oral Toxicity Test Study Code: 1003.1H.SAG22, Test Site Code: 22354-01R Renolab S.r.l. / SAGEA Centro di Saggio s.r.l. GLP Unpublished	N	Pestila* ProAgri**

*Pestila Spółka z ograniczoną odpowiedzialnością (short name: Pestila Sp. z o.o.)
**ProAgri Spółka z ograniczoną odpowiedzialnością or ProAgri International Spółka z ograniczoną odpowiedzialnością (short name: ProAgri Sp. z o.o. or ProAgri International Sp. z o.o.)

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

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

The following tables are to be completed by MS

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for diflufenican

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

Please refer to the points 5.2.1.1 and 5.2.1.2.

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

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

No new or additional studies have been submitted.

A 2.1.2.2 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)

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

A 2.1.2.7.1 HPLC - DAD (in sucrose solution)

A 2.1.2.7.1.1 Method validation

Comments of zRMS:	Method is accepted

Reference:	KCP 5.1.2/01 filled as KCP 10.3.1.1.1/02
Report	Diflufenikan 500 S.C. Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test, Kulec-Płoszczyca E., 2022, Study code: B-100-22
Guideline(s):	SANTE/2020/12830, Rev. 1 [1] and Standard Operating Procedure SOP/C/9
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The concentration of active substance of test item was chemically determined using the validated high performance liquid chromatographic method with DAD detection. The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical column:	Gemini C6-Phenyl 110Å 250 x 4.6
Wave length:	284 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : 0.05% ortho-phosphoric acid (85 : 15, v/v)
Flow rate:	0.8 mL/min
Detection System:	Diode Array Detector

Test item name:	Diflufenikan 500 SC
Batch no:	1/DIF/2022
Active substances:	diflufenican
CAS of active substance:	83164-33-4
Date of production:	01.2022
Expiry date:	01.2024

Working solutions

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

Fortification samples
For the preparation of procedural recoveries and validation experiments, fortification samples were pre-

pared from standard solutions. The appropriate amount standard solutions was added to the matrix to prepare LOQ and 10xLOQ. Fortification samples were prepared and analyzed to ensure the result fits within the range of the respective standard curve.

Sample preparation for the chromatographic analysis

First, 1 g sucrose solution was weighted into a volumetric flask with a capacity of 10 mL and was made volume up to 10 ml with mixture of acetonitrile : deionized water (50:50 v/v). Finally, extract was introduced into a chromatographic column.

Validation

Selectivity and specificity

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

Linearity

Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph and the ranges of calibration curve corresponding to the real samples level are presented in the table below.

Analyte	**range of linearity of calibration curve [mg/L]**	**equivalent calibration range of linearity [mg analyte/L]**
diflufenican	0.5 – 50.0	0.5 – 50.0

The equation of the calibration lines were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	**Slope**	**Intercept**	**Coefficient r^2**
diflufenican	48884.5	-1091.72	0.9999323

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

A summary of the recovery data of control and fortified samples are presented in the table below.

Analyte	**matrix**	**Fortification Level**	**Number of Replicates**	**Mean Recovery [%]**	**±**	**RSD [%]**
diflufenican	sucrose solution	Control	2	-	-	-
		LOQ	5	103.3	±	0.4

		10xLOQ	5	102.9	±	1.5

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analyzed is presented in table below. The RSD is ≤ 20% per each level.

Matrix Effect
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \frac{\text{peak area (matrix)}}{\text{peak area (solvent)}} - 100$$

The matrix effect is not exceed ± 20 %. The matrix effect and concentration are presented in table below.

Analyte	**matrix**	**Concentration mg/L**	**matrix effect [%]**
diflufenican	sucrose solution	1.0	-0.8

Limit of detection (LOD) and limit of quantification (LOQ)
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%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below.

LOQ	**equivalent calibration level**	**LOD**	**equivalent calibration level**
10.0 mg diflufenican/kg	1.0 mg/L	5.0 mg diflufenican/L	0.5 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the test item in matrix (sucrose solution) for Bumblebees.

A 2.1.2.7.2 HPLC - DAD (in 1% solution of triton in water)

A 2.1.2.7.2.1 Method validation

Comments of zRMS:	Method is accepted

Reference:	KCP 5.1.2/02 filled as KCP 10.3.1.1.2/02
Report	Diflufenikan 500 S.C. Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test, Kulec-Płoszczyca E., 2022, Study code: B-102-22
Guideline(s):	SANTE/2020/12830, Rev. 1 [1] and Standard Operating Procedure SOP/C/9
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The concentration of active substance of test item was chemically determined using the validated high performance liquid chromatographic method with DAD detection [SOP/C/59, SOP/C/304]. The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence (Shimadzu Corporation Japan)
Analytical column:	Gemini C6-Phenyl 110Å 250 x 4.6
Wave length:	284 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : 0.05% ortho-phosphoric acid (85 : 15, v/v)
Flow rate:	0.8 mL/min
Detection System:	Diode Array Detector

Test item name:	Diflufenikan 500 SC
Batch no:	1/DIF/2022
Active substances:	diflufenican
CAS of active substance:	83164-33-4
Date of production:	01.2022
Expiry date:	01.2024

Working solutions

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

Fortification samples
For the preparation of procedural recoveries and validation experiments, fortification samples were prepared from standard solutions. The appropriate amount standard solutions was added to the matrix to prepare LOQ and 10xLOQ. Fortification samples were prepared and analyzed to ensure the result fits within the range of the respective standard curve.

Sample preparation for the chromatographic analysis

Each sample in an appropriate amount was collected and applied to the chromatographic column in a volume of 20 µL. The sample was diluted with mixture of acetonitrile : deionized water (50:50 v/v), (if necessary).

Validation

Selectivity and specificity

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

Linearity

Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph and the ranges of calibration curve corresponding to the real samples level are presented in the table below.

Analyte	**range of linearity of calibration curve [mg/L]**	**equivalent calibration range of linearity [mg analyte/L]**
diflufenican	0.5 – 50.0	0.5 – 50.0

The equation of the calibration lines were presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 must be higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	**Slope**	**Intercept**	**Coefficient r^2**
diflufenican	48884.5	-1091.72	0.9999323

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

A summary of the recovery data of control and fortified samples are presented in the table below.

Analyte	**matrix**	**Fortification Level**	**Number of Replicates**	**Mean Recovery [%]**	**±**	**RSD [%]**
diflufenican	1% solution of triton in water	Control	2	-	-	-
		LOQ	5	103.0	±	0.4
		10xLOQ	5	105.6	±	0.1

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analyzed is presented in table below. The RSD is ≤ 20% per each level.

Matrix Effect
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \frac{\text{peak area (matrix)}}{\text{peak area (solvent)}} - 100$$

The matrix effect is not exceed ± 20 %. The matrix effect and concentration are presented in table below.

Analyte	**matrix**	**Concentration mg/L**	**matrix effect [%]**
diflufenican	1% solution of triton in water	1.0	2.2

Limit of detection (LOD) and limit of quantification (LOQ)
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%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below.

LOQ	**equivalent calibration level**	**LOD**	**equivalent calibration level**
1.0 mg diflufenican/L	1.0 mg/L	0.5 mg diflufenican/L	0.5 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the test item in matrix (1% solution of triton in water) for Bumblebees.

A 2.1.2.7.3 HPLC - DAD (in sediment and Smart&Barko medium)

A 2.1.2.7.3.1 Method validation

Comments of evaluator:	Method is accepted

Reference: KCP 5.1.2/03 (filed as KCP 10.2.1.3/02)

Report	Diflufenikan 500 SC Water-sediment *Myriophyllum spicatum* toxicity test, Study code: W-06-22, Czarnecka, M., 2022
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical methods were developed for the determination of active substance of test item in matrices. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Reagents, solvents, solutions and chemicals

- Deionized water, HPLC grade, Łukasiewicz-IPO, Fresh prepared before analysis
- Methanol, pure p.a., POCH, batch no. 1158/11/20
- Acetone, pure p.a., POCH, batch no. 1111/07/21
- SPE cartridges, Supelclean ENVI-18, SUPELCO, batch no. 12546401
- Acetonitrile, HPLC grade, POCH and VWR Chemicals, batch no. 1171/08/21, 1039/11/21, 1375/03/22, 22E194018
- Hydrochloric acid 37%, ACS reagent, Sigma-Aldrich, batch no. STBK 0854
- Ortho-phosphoric acid 85% pure p.a., Supelco, batch no. Z0721828108
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile : deionized water (50:50 v/v),
- standard solution of 1.0 mg/mL of diflufenican in acetonitrile for HPLC and working solution at concentration 100, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL in mixture of acetonitrile and deionized water (50:50 v/v).

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Balance, WPS 510/C, ZMP RADWAG (Poland)
- Laboratory centrifuge, MPW-351e, MPW Med. Instruments
- Ultrasonic bath, Sonic-5, Polsonic
- SPE vacuum manifold, Visiprep, Supelco (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Various volumes, Brand (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Gemini C6-Phenyl 110Å 250 x 4.6
- Oven temperature: 35°C
- Flow Rate: 0.80 mL/min
- Wavelength: 228420 nm
- Injection volume: 20 µL
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (85 : 15, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Sediment phase of water-sediment system

First, 5 mL of mixture acetonitrile and deionized water (50:50; v/v), was added to 10 g of sediment sample and shaken for 2 minutes and sonicated for 10 minutes. The sample was centrifuged and filtered through filter paper. Then extraction was repeated with 5 mL of mixture acetonitrile and deionized water (50:50; v/v). Finally, an aliquot of the mixed extract was transferred into a HPLC vial for further quantification using HPLC-DAD. The sample was diluted with mixture of acetonitrile and deionized water (50:50 v/v) (if necessary).

Water phase of water-sediment system - Smart&Barko medium

Each sample of 100 mL volume was acidified by hydrochloric acid to pH≤2 and applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of acetone, twice with 5 mL of methanol, twice with 5 mL of deionized water pH≤2. Following the sample introduction the column was dried under vacuum for 5 minutes. The active substance was eluted with 5 mL of acetone and 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 deionized water (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation

Selectivity and specificity

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

Matrix effect

Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.

Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

The matrix effect is not exceed ± 20 %.

Analyte	Slope	Concentration mg/L	matrix effect [%]
diflufenican	sediment	0.05	-7.0
diflufenican	Smart&Barko medium	0.05	4.9

Linearity

Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded.

Analyte	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg analyte/L]
diflufenican	0.01 – 1.0	0.0001 – 0.01

The equation of the calibration line was presented as the linear equation; y = ax + b (a – slope, b - inter-

cept). The linear coefficient r2 is higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient r^2
diflufenican	47651.0	94.4910	0.9999918

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation.
Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

Analyte	Matrix	Fortification Level	Number of Replicates	Mean Recovery [%]		RSD [%]
diflufenican	sediment	Control	2	-	-	-
		LOQ	5	97.4	±	8.2
		10xLOQ	5	102.6	±	0.8
diflufenican	Smart&Barko medium	Control	2	-	-	-
		LOQ	5	110.8	±	2.0
		10xLOQ	5	108.0	±	1.3

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
sediment	0.05 mg diflufenican/kg	0.05 mg/L	0.01 mg diflufenican/kg	0.01 mg/L
Smart&Barko medium	0.0005 mg diflufenican/L	0.05 mg/L	0.0001 mg diflufenican/L	0.01 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in sediment and Smart&Barko medium.

A 2.1.2.7.4 HPLC - DAD (in Elendt M7 medium)

A 2.1.2.7.4.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/04 (filed as KCP 10.2.1.3/01)
Report	Diflufenikan 500 SC *Daphnia magna*, Acute Immobilisation Test, Study code: W-07-22, Czarnecka, M., 2022
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical methods were developed for the determination of active substance of test item in matrices. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Reagents, solvents, solutions and chemicals

- Deionized water, HPLC grade, Łukasiewicz-IPO, Fresh prepared before analysis
- Acetonitrile, HPLC grade, POCH, batch no. 1171/08/21, 1078/09/21, 0314/11/21
- Ortho-phosphoric acid 85% pure p.a., Supelco, batch no. Z0721828108
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile : deionized water (50:50 v/v),
- standard solution of 1.0 mg/mL of diflufenican in acetonitrile for HPLC and working solution at concentration 100, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL in mixture of acetonitrile and deionized water (50:50 v/v).

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Various volumes, Brand (Germany)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Gemini C6-Phenyl 110Å 250 x 4.6
- Oven temperature: 35°C
- Flow Rate: 0.80 mL/min
- Wavelength: 284 nm
- Injection volume: 20 µL
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (85 : 15, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample in an appropriate amount was collected and applied to the chromatographic column in a volume of 20 µL. The sample was diluted with mixture of acetonitrile : deionized water (50:50 v/v), (if necessary).

Validation

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

Matrix effect
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

The matrix effect is not exceed ± 20 %.

Analyte	Slope	Concentration mg/L	matrix effect [%]
diflufenican	Elendt M7 medium	0.05	3.5

Linearity
Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded.

Analyte	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg analyte/L]
diflufenican	0.5 – 50.0	0.5 – 50.0
diflufenican	0.01 – 1.0	0.01 – 1.0

The equation of the calibration line was presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 is higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient r^2
diflufenican	48884.5	-1091.72	0.9999323
diflufenican	47651.0	94.4910	0.9999918

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation.
Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

Analyte	Matrix	Fortification Level	Number of Replicates	Mean Recovery [%]		RSD [%]
diflufenican	Elendt M7 medium	Control	2	-	-	-
		LOQ	5	90.8	±	10.8
		10xLOQ	5	87.0	±	4.4

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
Elendt M7 medium	0.05 mg diflufenican/L	0.05 mg/L	0.01 mg diflufenican/L	0.01 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in Elendt M7 medium.

A 2.1.2.7.5 HPLC-MS/MS (in AAP medium)

A 2.1.2.7.5.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/05 (filed as KCP 10.2.1.3/01)
Report	Diflufenikan 500 SC *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test, Study code: W-08-22, Czarnecka, M., 2022
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 diflufenican in AAP 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 chromatographic method with MS/MS detection.

Reagents, solvents and chemicals

- Water, deionized (LC-MS), Fresh prepared before analysis
- AAP medium, Fresh prepared before analysis

- Methanol, LC-MS, POCH, batch no. 0818/09/20
- Acetone, pure p.a., POCH, batch no. 1316/01/22
- Acetonitrile, LC-MS, POCH, batch no. 2125005850
- SUPELCLEAN ENVI-18 SPE, 3 mL, 500 mg, Supelco, batch no. 12878002
- Hydrochloric acid, ACS 37%, batch no. STBK0854
- Formic acid, ≥99%, VWR Chemicals, batch no. PW743391
- mixture of acetonitrile LC-MS : formic acid LC-MS (1000:1, v/v),
- mixture of deionised water : formic acid LC-MS (1000:1, v/v),
- deionised water acidified by hydrochloric acid to pH ≈ 2,
- standard solution of 1 mg/mL of diflufenican in acetonitrile for LC-MS,
- working solution of diflufenican at concentration 10.0 and 0.1 µg/mL in mixture of acetonitrile LC-MS and formic acid (1000:1, v/v),
- calibration solutions of diflufenican at concentration 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 µg/mL in AAP matrix extract.

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Variable volume, Eppendorf AG (Germany)
- SPE vacuum manifold, Visiprep, Supelco (USA)
- SPE cartridges, Supelclean ENVI-18, Supelco (USA)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Chromatograph, Prominence-*i*, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: Shimadzu Nexera XR
- Analytical column: Kinetex 2.6µm C18 100A, l=50 mm, Ø=2.1 mm
- Column temperature: 35°C
- Flow Rate: 0.5 mL/min
- Injection volume: 2 µl
- Mobile Phase A: Water : Formic acid (1000 : 1, v/v)
- Mobile Phase B: Acetonitrile
- Gradient (including wash and equilibration):

Time [min]	Phase A [%]	Phase B [%]
0.00	90	10
0.50	90	10
1.50	5	95
2.20	5	95
2.22	90	10
4.50	90	10

- Detection System: Shimadzu LCMS-8045 Mass Spectrometer
- Ionisation: Electro Spray (ESI)
- Analyte: Diflufenican
- Transitions: 395.00 --> 265.90[1)]
  395.00 --> 246.05[2)]
- Polarity: positive

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

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was acidified by hydrochloric acid to pH ≈ 2 and applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing with 10 mL of acetone pure, 10 mL

methanol for LC-MS and with 10 mL of deionised water acidified by hydrochloric acid to pH ≈ 2. Following the sample introduction the column was dried under vacuum for 5 minutes. The active substance was eluted with 10 mL of methanol for LC-MS and 5 mL of acetone. The eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was redissolved in mixture of acetonitrile LC-MS : formic acid LC-MS (1000:1, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC with MS/MS detection.

Validation

Selectivity and 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 was 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.

Matrix effect

Since potential matrix effects were compensated by using matrix matched calibration standards (same matrix load), no instrument recovery samples were prepared and analysed in addition.

Linearity

Working solutions of diflufenican at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.05 ng/mL to 5 ng/mL. The range of calibration curve of diflufenican is equivalent to range from 0.0025 µg diflufenican /L to 0.25 µg diflufenican /L in AAP medium.

The equations of the calibration line are presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r2 is higher than 0.99. Range of the linear was given in ng/mL equivalent to µg/L.

Analyte	Transitions	Slope	Intercept	Coefficient r^2
Diflufenican	Quantitation Transition 395.00 → 265.90	66599.62	26313.20	0.9996209
	Confirmatory Transition 395.00 → 246.05	5642.823	2217.288	0.9994491

Linear weighted calibration (1/x weighting) is used. The suitability of the chosen function was demonstrated as the regression residual (di).

The calibration curves above were used to calculations containing detected substance in a fortification samples.

Before each analyses the working solutions of diflufenican at the 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were injected successively to the chromatographic column and the chromatograms were recorded. The results generated during the definitive experiment was calculated on base the two different calibration curves. All standard curves used in definitive experiment (peak area versus quantity of the standard) are linear. The range of linearity of the analytical graphs are from 0.05 ng/mL to 5.0 ng/mL.

The equation of the calibration line is presented as the linear equation; y = ax + b (a –slope, b - intercept). The linear coefficient r2 is higher than 0.99. Range of the linear was given in ng/mL.

Analyte	Date of analysis	Slope	Intercept	Coefficient r^2
Diflufenican	18.07.2022	36591.2	46896.5	0.9969732
	21.07.2022	37789.2	3935.93	0.9998622

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels of diflufenican appropriate to level corresponding with LOQ and 10

x LOQ. Mean recoveries ± relative standard deviation (RSD) for each level is in the range 70-120%.

Active substance	Matrix	Transitions	Fortification Level [µg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Diflufenican	AAP medium	Quantitation Transition 395.00 → 265.90	0.01	5	93.4	10.8
			0.10	5	95.2	1.9
		Confirmatory Transition 395.00 → 246.05	0.01	5	91.4	13.7
			0.10	5	96.8	3.9

In order to study the recovery level, the solution of the detected substance were added to non-treated AAP medium and then analyzed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
AAP medium	0.01 mg diflufenican/L	0.2 mg/L	0.0025 mg diflufenican/L	0.05 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in APP medium.

A 2.1.2.7.6 HPLC with DAD detection (in water)

A 2.1.2.7.6.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/06 (filed as KCP 10.2.1.4/01)
Report	Diflufenikan 500 SC *Lemna gibba* CPCC 310, Growth inhibition test, Study code: W-09-22, Czarnecka, M., 2022
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 the active substance of the test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Reagents, solvents and chemicals

- Water, deionized, HPLC grade, Fresh prepared before analysis
- SPE cartridges, Supelclean ENVI-18, SUPELCO, batch no. 12546401, 12878002
- Methanol, pure p.a, POCH, batch no. 1158/11/20
- Acetone, pure p.a., POCH, batch no. 1111/07/21
- Acetonitrile, HPLC grade, POCH, batch no. 0314/11/21, 1078/09/21
- Hydrochloric acid 37%, pure p.a, batch no. STBK0854
- Ortho-phosphoric acid 85%, pure p.a, Supelco, batch no. Z0721828108
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile : deionized water (50:50 v/v),
- standard solution of 1.0 mg/mL of diflufenican in acetonitrile for HPLC and working solution at concentration 100, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL in mixture of acetonitrile and deionized water (50:50 v/v)

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Variable volume, Eppendorf AG (Germany)
- SPE vacuum manifold, Visiprep, Supelco (USA)
- Rotary vacuum evaporator with water bath, RV 05 basic, HB 4 basic, IKA - WERKE (Germany)
- Rotary vacuum evaporator with water bath, RV 10 digital, HB 10 digital, IKA - WERKE (Germany)
- Chromatograph, Prominence-*i*, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Gemini C6-Phenyl 110Å 250 x 4.6
- Oven temperature: 35°C
- Flow Rate: 0.80 mL/min
- Wavelength: 284 nm
- Injection volume: 20 µL
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (85 : 15, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was acidified by hydrochloric acid to pH≤2 and applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing twice with 5 mL of acetone, twice with 5 mL of methanol, twice with 5 mL of deionised water pH≤2. Following the sample introduction the column was dried under vacuum for 5 minutes. The active substance was eluted with 5 mL of acetone and 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 deionized water (50:50; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation

Selectivity and specificity

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

Matrix effect

Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.

Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

The matrix effect is not exceed ± 20 %.

Analyte	Slope	Concentration mg/L	Matrix effect [%]
diflufenican	water	0.05	9.7

Linearity

Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded.

Analyte	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg analyte/L]
diflufenican	0.5 – 50.0	0.005 – 0.5
diflufenican	0.01 – 1.0	0.0001 – 0.01

The equation of the calibration line was presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 is higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient r^2
diflufenican	48884.5	-1091.72	0.9999323
diflufenican	47651.0	94.4910	0.9999918

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation.

Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

Analyte	Matrix	Fortification Level	Number of Replicates	Mean Recovery [%]		RSD [%]
diflufenican	water	Control	2	-	-	-
		LOQ	5	104.4	±	2.3
		10xLOQ	5	103.2	±	1.2

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
water	0.0005 mg diflufenican/L	0.05 mg/L	0.0001 mg diflufenican/L	0.01 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in water.

A 2.1.2.7.7 HPLC with DAD detection (in artificial soil)

A 2.1.2.7.7.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/07 (filed as KCP 10.4.1.1/01)
Report	Diflufenikan 500 SC Earthworm reproduction test *(Eisenia andrei)*, Study code: G-89-21, Pieczka, P., 2022
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 the active substance of the test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Reagents, solvents and chemicals

- Water, deionized, HPLC grade, Fresh prepared before analysis
- Acetonitrile, HPLC grade, POCH, batch no. 1078/09/21, 0314/11/21, 1039/11/21
- Ortho-phosphoric acid 85%, pure p.a, Supelco, batch no. Z0721828108

- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile : deionized water (50:50 v/v),
- standard solution of 1.0 mg/mL of diflufenican in acetonitrile for HPLC and working solution at concentration 100, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL in mixture of acetonitrile and deionized water (50:50 v/v)

Equipment
- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Balance, WPS 510/C, Radwag (Poland)
- Laboratory centrifuge, MPW-351e, MPW Med. Instruments
- Ultrasonic bath, Sonic-5, Polsonic
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Variable volume, Eppendorf AG (Germany)
- Chromatograph, Prominence-*i*, Shimadzu Corp. (Japan)

Chromatographic conditions
- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Gemini 3µm C6-Phenyl 110Å 250 mm x 4.6 mm
- Oven temperature: 35°C
- Flow Rate: 0.80 mL/min
- Wavelength: 284 nm
- Injection volume: 20 µL
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (85 : 15, v/v)
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
First, 5 mL of mixture acetonitrile and deionized water (50:50; v/v), was added to 10 g of artificial soil sample and shaken for 2 minutes and sonicated for 10 minutes. The sample was centrifuged and filtered through folter paper. Then extraction was repeated with 5 mL of mixture acetonitrile and deionized water (50:50; v/v). Finally, an aliquot of the mixed sample was diluted with mixture acetonitrile and deionized water (50:50; v/v) (if necessary).

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

Matrix effect
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

The matrix effect is not exceed ± 20 %.

Analyte	Slope	Concentration mg/L	Matrix effect [%]
diflufenican	Artificial soil	1.0	-7.7

Linearity
Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded.

Analyte	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg analyte/L]
diflufenican	0.5 – 50.0	5 – 500

The equation of the calibration line was presented as the linear equation; y = ax + b (a – slope, b - intercept). The linear coefficient r^2 is higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient r^2
diflufenican	48884.5	-1091.72	0.9999323

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation.
Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.

Analyte	Matrix	Fortification Level	Number of Replicates	Mean Recovery [%]		RSD [%]
diflufenican	Artificial soil	Control	2	-	-	-
		LOQ	5	81.0	±	1.9
		10xLOQ	5	76.8	±	1.0

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
Artificial soil	10.0 mg diflufenican/kg	1.0 mg/L	5.0 mg diflufenican/kg	0.5 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in artificial soil.

A 2.1.2.7.8 HPLC with DAD detection (in water)

A 2.1.2.7.8.1 Method validation

Comments of evaluator:	Method is accepted

Reference:	KCP 5.1.2/08 (filed as KCP 10.6.2/02)
Report	Diflufenikan 500 SC Terrestial Plant Test: Vegetative Vigour Test, Study code: G-91-21, Pieczka, P., 2022
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

This method is also suitable for study code: G-92-21 (Diflufenikan 500 SC Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, Pieczka, P., 2022)

Materials and methods

The analytical method was developed for the determination of the active substance of the test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, matrix effect, accuracy, and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Reagents, solvents and chemicals

- Water, deionized, HPLC grade, Fresh prepared before analysis
- Acetonitrile, HPLC grade, POCH, batch no. 1171/08/21, 1039/11/21
- Ortho-phosphoric acid 85%, pure p.a, Supelco, batch no. Z0721828108
- 0.05% ortho-phosphoric acid solution in deionized water,
- mixture of acetonitrile : deionized water (50:50 v/v),
- standard solution of 1.0 mg/mL of diflufenican in acetonitrile for HPLC and working solution at concentration 100, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL in mixture of acetonitrile and deionized water (50:50 v/v)

Equipment

- Balance, Adventurer Pro AV 114CM, Ohaus Corporation (USA)
- Volumetric flasks, Various volumes, Glassco (Germany)
- Automatic pipettes, Variable volume, Eppendorf AG (Germany)
- Chromatograph, Prominence, Shimadzu Corp. (Japan)

Chromatographic conditions

- Chromatographic System: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence (Shimadzu Corporation Japan)
- Analytical column: Gemini 3µm C6-Phenyl 110Å 250 mm x 4.6 mm
- Oven temperature: 35°C
- Flow Rate: 0.80 mL/min
- Wavelength: 284 nm
- Injection volume: 20 µL
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid, (85 : 15, v/v)

- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
Each sample in an appropriate amount was collected and applied to the chromatographic column in a volume of 20 µL. The samples were diluted with mixture acetonitrile and deionized water (50:50; v/v).

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

Matrix effect
Matrix effect for each method was checked during the method validation. Assessment of matrix effects was performed by comparing the standard preparing in solution to standard preparing in blank matrix at appropriate concentration.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

$$\text{Matrix effects [\%]} = 100 \times \text{peak area (matrix)/peak area (solvent)} - 100$$

The matrix effect is not exceed ± 20 %.

Analyte	Slope	Concentration mg/L	Matrix effect [%]
diflufenican	water	0.05	-7.0

Linearity
Working solutions at all calibration levels were injected successively to the chromatographic column and the chromatograms were recorded.

Analyte	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg analyte/L]
diflufenican	0.5 – 50.0	0.5 – 50.0
diflufenican	0.01 – 1.0	0.01 – 1.0

The equation of the calibration line was presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 is higher than 0.99. Range of the linear range was given, e.g. mg/L (equal to µg/mL).

Analyte	Slope	Intercept	Correlation coefficient r^2
diflufenican	48884.5	-1091.72	0.9999323
diflufenican	47651.0	94.4910	0.9999918

Linear weighted calibration (1/C weighting) is used for calibration curve. The suitability of the chosen function were demonstrated as the regression residual (di). The regression residual is presented in a residual plot in range equal to range of linearity of calibration curve.

Accuracy
The accuracy of the method is reported as mean recovery ± relative standard deviation.
Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each

level is in the range 70-120%.

Analyte	Matrix	Fortification Level	Number of Replicates	Mean Recovery [%]		RSD [%]
diflufenican	water	Control	2	-	-	-
		LOQ	5	102.0	±	2.7
		10xLOQ	5	84.0	±	17.6

In order to study the recovery level, the solutions of the detected substances were added to non-treated water samples and then analysed using the method described above.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The RSD is ≤ 20% per each level.

Limit of detection and limit of quantification
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

matrix	LOQ	equivalent calibration level	LOD	equivalent calibration level
water	0.05 mg diflufenican/L	0.05 mg/L	0.01 mg diflufenican/L	0.01 mg/L

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance diflufenican of the test item Diflufenikan 500 SC in water.