

FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: Acetamipryd 200 SL

Product name(s): -

Chemical active substance:

acetamiprid, 200 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: March 2024

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Version history

When	What
February 2025	zRMS assessment of dRR
August 2025	The final Registration Report after the reporting period.

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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.

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

Commodity/crop	Supported/ Not supported
Winter oilseed rape, Spring oilseed rape, Turnip rape	Supported
Potatoe	Supported
Apple, Wild apple	Supported
Pear, Chinese pear	Supported
Quince	Supported
Medlar	Supported
Plum	Supported
Peach	Supported
Nectarine	Supported
Apricot	Supported
Sour cherry, Sweet cherry	Supported
Tomato	Supported
Aubergine/eggplant	Supported
Pepper	Supported
Walnut	Supported
Hazelnut	Supported

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

5.2.1.1 Analysis of the plant protection product (KCP 5.1.1)

5.2.1.2 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 acetamiprid in plant protection product is provided as follows:

Comments of zRMS:	The proposed analytical method is suitable for the determination of active substance acetamiprid in the plant protection product Piorun 200 SL.
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	The proposed analytical method has been fully validated in terms of specificity, linearity, repeatability, and recovery. Proposed method and its validation fulfil the requirements of SANCO/3030/99 rev. 5 guidance. The method and its validation are accepted.
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Reference: 5.1.1/01
5.1.1/02

Report Acetamipryd 200 SL. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage.
Kupiec J., 2022, report no. BF – 23/22

Guideline(s): Yes, SANCO/3030/99 rev.5 (22/03/19)

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Determination of acetamiprid in Acetamipryd 200 SL was performed with using reversed phase high performance liquid chromatography (RP-HPLC) with UV-Vis detection at wavelength 246 nm.

Equipment and chromatographic conditions for prothioconazole analysis

- Shimadzu liquid chromatograph equipped with UV-Vis detector
- Column: Luna Omega, 250x4.6mm, 5µm
- Analytical balance Mettler Toledo AT261, accuracy 0.01 mg
- Glass pipettes
- Volumetric flasks
- Autosampler vials
- Ultrasonic bath, POLSONIC
- Typical laboratory equipment
- Analytical standards
- Deionized water, ultra-pure, Millipore
- Acetonitrile for HPLC, POCh
- Oven temperature: 35°C
- Flow rate: 1 ml/min
- Wavelength $\lambda = 246$ nm
- Injection volume: 3 µl
- Mobile phase composition: acetonitrile + H₂O (28+72) (v/v)

Under the above conditions the retention time for Acetamiprid is 8.6 min ± 0.3min. Total time of analysis is 15 min.

The preparation of standard solution

60.86 mg of Acetamiprid 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 30 mg of examined specimen was weighed (with the accuracy of 0.01 mg) into the 10 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume and analyzed.

The preparation of placebo solution

371.47 mg of placebo was weighed (with the accuracy of 0.0 mg) into the 25 ml volumetric flask. Acetonitrile was added, stirred and the flask was put into the ultrasonic bath (5 min). After cooling, acetonitrile was added to the nominal volume. Solutions was diluted and analyzed.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances acetamiprid in plant protection product Acetamipryd 200 SL

Acetamiprid	
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 acetamiprid standard solutions in the concentration range from 0.3644 mg/mL to 0.9111 mg/mL (from 70% to 164% of the declared content). Correlation coefficient: $R^2 = 0.997$ Required: $R^2 \geq 0.99$
Precision – Repeatability Mean n = 6 (%RSD)	Hr = 0.26 Required: $Hr \leq 1$ RSD = 0.46 Required: $RSD \leq 1.74$
Accuracy n = 12 (2 levels) (% Recovery)	Total recovery 101.33% (range: 100.2% - 102.4%) Required: 97% - 103%
Interference/ Specificity	Fulfilled. Superimposed chromatograms of: solvent, placebo, standard, and formulation were included.
Comment	No comments.

Conclusion

The reversed phase high performance liquid chromatography (RP-HPLC) with UV-Vis detection, used to quantify acetamiprid in Acetamipryd 200 SL was fully validated. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

Not relevant. The product Acetamipryd 200 SL does not contain relevant impurities.

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

Not relevant. The product Acetamipryd 200 SL does not contain materials of toxicological, ecotoxicological or environmental concern.

5.2.1.5 Applicability of existing CIPAC methods (KCP 5.1.1)

For acetamiprid soluble concentrates CIPAC Method (649/SL/(M)/) is suitable. The guideline ‘Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Regulation (EU) 283/2013 and Regulation (EU) 545/2011 of Regulation 1107/2009/EC’ states: ‘The applicability of existing CIPAC methods shall be assessed and reported. In case of use of a CIPAC method, further validation data shall not be required, but example chromatograms shall be submitted, where available.

5.2.1.6 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 acetamiprid 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: acetamiprid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Sucrose solution <i>Bumblebees (Bombus spp.)</i> , Acute Oral Toxicity Test (Ecotoxicology)	Primary	5 mg/kg	HPLC-DAD	Fulczyk A., 2022, Report No. B-105-22
1% Triton water solution <i>Bumblebees (Bombus spp.)</i> , Acute Contact Toxicity Test (Ecotoxicology)	Primary	50 mg/L	HPLC-DAD	Fulczyk A., 2022, Report No. B-107-22
Water <i>Raphidocelis subcapitata</i> Growth inhibition test (Ecotoxicology)	Primary	0.06 mg/L	HPLC-DAD	Czarnecka, M., 2022, Report No. W-12-22
Eelndt M7 medium <i>Daphnia magna</i> , Acute Immobilisation Test (Ecotoxicology)	Primary	0.06 mg/L	HPLC-DAD	Czarnecka, M., 2022, Report No. W-11-22
Water Vegetative Vigour Test (Ecotoxicology)	Primary	0.06 mg/L	HPLC-DAD	Wróbel, A., 2022, Report No. G-95-21
Water Seedling Emergence and Seedling Growth Test (Ecotoxicology)	Primary	0.06 mg/L	HPLC-DAD	Wróbel, A., 2022, Report No. G-96-21
Artificial soil Earthworm reproduction test (<i>Eisenia andrei</i>) (Ecotoxicology)	Primary	0.05 mg/kg	HPLC-DAD	Wróbel A., 2022, Report No. G-93-21
Sucrose solution Honeybees (<i>Apis mellifera</i> L.), Chronic Oral Toxicity	Primary & confirmatory	10 mg/kg	HPLC tandem LC-MS/MS	Morsiani S., 2024, Report No. 23128-03R; 1032.1I.SAG23

Component of residue definition: acetamiprid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Test (Ecotoxicology)				
Water Honeybees (<i>Apis mellifera</i> L.), Larval Toxicity Test Following Repeated Exposure (Ecotoxicology)	Primary & confirmatory	0.00052 g/L	HPLC tandem LC-MS/MS	Mautino G., 2024, Report No. 23128-04R; 1033.1I.SAG23
Soil <i>Hypoaspis (Geolaelaps) aculeifer</i> reproduction test (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg (referred to dry soil)	HPLC tandem LC-MS/MS	Mautino G., 2023, Report No. 23128-01R; 1039.1I.SAG23
Oilseed rape - seed and plant (Residues)	Primary & confirmatory	0.005 mg/kg for acetamiprid 0.005 mg/kg for N-desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) expressed as acetamiprid	LC-MS/MS	Stasiak S., 2023, Validation Study No: VAL/10/2023
Potato (Residues)	Primary & confirmatory	0.005 mg/kg for acetamiprid 0.005 mg/kg for N-desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) expressed as acetamiprid	LC-MS/MS	Niewelt-Stasiak S., 2023, Validation Study No: VAL/11/2023
Apple - fruit (Residues)	Primary & confirmatory	0.005 mg/kg for acetamiprid 0.005 mg/kg for N-desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) expressed as acetamiprid	LC-MS/MS	Niewelt-Stasiak S., 2023, Validation Study No: VAL/12/2023
Artificial soil	Primary &	4 µg/kg	UHPLC-	Szlauer S., 2024, Study

Component of residue definition: acetamiprid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Collembolan (<i>Folsomia candida</i>) - Reproduction Test in soil (Ecotoxicology)	confirmatory		MS/MS	code: ETOX-2024-2

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

Component of residue definition: Sum of acetamiprid and metabolite IM-2-1 (N-desmethyl-acetamiprid), expressed as acetamiprid				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Honey (Residues)	Primary & confirmatory	0.005 mg/kg for acetamiprid 0.005 mg/kg for N-desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1)	LC-MS/MS	Lefebvre C., 2023 Report No. R C2051

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

5.3.1.1 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 5.2.1.1.

5.3.1.2 Description of analytical methods for the determination of residues acetamiprid (KCP 5.2)

5.3.1.3 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	Acetamiprid	0.01 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Plant, high acid content		0.01 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Plant, high oil content		0.01 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Muscle	N-desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid	0.02 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Milk		0.2 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Eggs		0.02 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Fat		0.02 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Liver, kidney		0.02 mg/kg	Reg. (EU) 2019/88 Reg. (EU) 2025/158
Soil (Ecotoxicology)	Acetamiprid	0.05 mg/kg or xxx mg/kg	common limit
Drinking water (Human toxicology)	Acetamiprid and IM-1-5	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Acetamiprid	EC ₅₀ : 0.0207 mg a.s./L	<i>Chironomus riparius</i> EFSA Journal 2016;14(11):4610
Air	Acetamiprid	7.5 µg/m ³ AOEL sys: 0.025 mg/kg bw/d	SANTE/2020/12830, Rev.2 14. February 2023
Tissue (meat or liver)	No residue definition provided, IM-2-1 and 6-chloronicotinic acid (IC-0) were the main residues identified in rat urine.	0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023
Body fluids		0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023

5.3.1.4 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 acetamiprid in plant matrices is given in the following tables.

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: acetamiprid

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	Schwarz, T., 2008, Study No. RD-01937, EU agreed, Netherlands, RAR, 2015 Weber, H., 2013, Study No. RD-02603, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Giseau, A., and Weber, H., 2012, Study No. RD-02454, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	Not required.		
High acid content	Primary	0.01 mg/kg	HPLC-MS/MS	Schwarz, T., 2008, Study No. RD-01937, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Giseau, A., and Weber, H., 2012, Study No. RD-02454, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	Not required.		
High oil content	Primary	0.01 mg/kg	HPLC-MS/MS	Schwarz, T., 2008, Study No. RD-01937, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Giseau, A. and Weber, H., 2012, Study No. RD-02454, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	Not required.		
High protein/high starch content (dry)	Primary	0.01 mg/kg	HPLC-MS/MS	Schwarz, T., 2008, Study No. RD-01937, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Giseau, A., and Weber, H., 2012, Study No. RD-02454, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	Not required.		

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	DAR section B.7.1
Not required, because:	-

5.3.1.5 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 acetamiprid in animal matrices is given in the following tables.

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: <i>N</i> -desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid				
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	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Knoch, E., 2010, Study No. RD-02156, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	-	-	-
Eggs	Primary	0.01 mg/kg	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Knoch, E., 2010, Study No. RD-02156, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	-	-	-
Muscle	Primary	0.01 mg/kg	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Knoch, E., 2010, Study No. RD-02156, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	-	-	-
Fat	Primary	0.01 mg/kg	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Knoch, E., 2010, Study No. RD-02156, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	-	-	-
Kidney, liver	Primary	0.01 mg/kg	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
	ILV	0.01 mg/kg	HPLC-MS/MS	Knoch, E., 2010, Study No. RD-02156, EU agreed, Netherlands, RAR, 2015
	Confirmatory (if required)	-	-	-
Component of residue definition: sum of acetamiprid and <i>N</i> -desmethyl-acetamiprid (IM-2-1) expressed as acetamiprid				

Component of residue definition: <i>N</i> -desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid				
Matrix type	Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Matrix type	Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Honey	Primary & confirmatory	0.005 mg/kg for acetamiprid 0.005 mg/kg for <i>N</i> -desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and <i>N</i> -desmethyl-acetamiprid (IM-2-1)	LC-MS/MS	Lefebvre C., 2023, Report No. R C2051
	ILV	0.005 mg/kg for acetamiprid 0.005 mg/kg for <i>N</i> -desmethyl-acetamiprid (IM-2-1) 0.01 mg/kg for sum of acetamiprid and <i>N</i> -desmethyl-acetamiprid (IM-2-1)	LC-MS/MS	Niewelt-Stasiak S., 2024, Report No. ILV/02/2023
	Confirmatory (if required)	Included in primary method.		

Table 5.3-5: Statement on extraction efficiency

	Method for products of plant origin
Required, available from:	DAR section B.7.1
Not required, because:	-

5.3.1.6 Description of methods for the analysis of soil (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid 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

Component of residue definition: acetamiprid and its metabolite and IM-1-5			
Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.002 mg/kg (acetamiprid and its metabolite IM 1-5)	LC-MS/MS	Täufner, A. and Weber, H., 2010, Study No. RD-02062N , EU agreed, Netherlands, RAR, 2015

Component of residue definition: acetamiprid and its metabolite and IM-1-5			
Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing
Confirmatory	Not required.	-	-

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid 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

Component of residue definition: acetamiprid and its metabolite IM-1-5				
Matrix type	Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water	Primary	0.1 µg/L (acetamiprid)	HPLC-MS/MS	Miya, K., 2007, Study No. RD-01204, EU agreed, Netherlands, RAR, 2015
	ILV	0.1 µg/L (acetamiprid)	HPLC-MS/MS	Senciuc, M., 2014a, Study No. RD-02951, EU agreed, Netherlands, RAR, 2015
	Primary	0.05 µg/L (IM-1-5)	HPLC-MS/MS	Giesau, A., and Weber, H., 2012, Study No. RD-02604, EU agreed, Netherlands, RAR, 2015
	ILV	0.05 µg/L (IM-1-5)	HPLC-MS/MS	Senciuc, M., 2014b, Study No. RD-02952, EU agreed, Netherlands, RAR, 2015
	Confirmatory	Not required.	-	-
Surface water	Primary	0.1 µg/L (acetamiprid)	HPLC-MS/MS	Miya, K., 2007, Study No. RD-01204, EU agreed, Netherlands, RAR, 2015
	Primary	0.1 µg/L (IM-1-5)	HPLC-MS/MS	Giesau, A., and Weber, H., 2012, Study No. RD-02604, EU agreed, Netherlands, RAR, 2015
	Confirmatory	Not required.	-	-

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid 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

Component of residue definition: acetamiprid

Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.002 µg/m ³	HPLC-MS/MS	Beck, T., and Class, T., 2009, Study No. RD-01863, EU agreed, Netherlands, RAR, 2015
Confirmatory	Not required.	-	-

5.3.1.9 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 acetamiprid in body fluids and tissues is given in the following table. No new studies have been submitted with this application.

Table 5.3-9: Methods for body fluids and tissues

Component of residue definition: no residue definition provided, IM-2-1 and 6-chloronicotinic acid (IC-0) were the main residues identified in rat urine (EFSA Journal 2016;14(11):4610)			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.05 mg/L (in blood)	HPLC-MS/MS	Senciuc, M., 2014c, Study No. RD-02943, EU agreed, Netherlands, RAR, 2015
Primary	0.01 mg/kg (in muscle and liver and kidney)	HPLC-MS/MS	Miya, K., 2010, Study No. RD-02080, EU agreed, Netherlands, RAR, 2015
Confirmatory	Not required.		

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 1.

5.3.1.10 Other studies/ information

Not required.

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	Kupiec J.	2022	ACETAMIPRYD 200 SL. Stage I: Determination of physicochemical properties of the initial preparation, after accelerated and low temperature storage. Report No. BF – 23/22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry GLP Published	N	Pestila* ProAgri**
KCP 5.1.2/01 (filled as KCP 10.3.1.1.1/02)	Fulczyk A.	2022	Acetamipryd 200 SL, Bumblebees (<i>Bombus</i> spp.), Acute Oral Toxicity Test Study code: B-105-22 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)	Fulczyk A.	2022	Acetamipryd 200 SL Bumblebees (<i>Bombus</i> spp.), Acute Contact Toxicity Test Study code: B-107-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/03 (filed as KCP 10.2.1.3/01)	Czarnecka M.	2022	Acetamipryd 200 SL <i>Raphidocelis subcapitata</i> SAG 61.81 (formerly <i>Pseudokirchneriella subcapitata</i>), Growth inhibition test Study code: W-12-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP	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
			Unpublished		
	Czarnecka M.	2023	AMENDMENT NO. 1 TO THE FINAL REPORT Acetamipryd 200 SL <i>Raphidocelis subcapitata</i> SAG 61.81 (formerly <i>Pseudokirchneriella subcapitata</i>), Growth inhibition test Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Poland Study code: W-12-22 GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/04 (filed KCP 10.2.1.2/01)	Czarnecka M.	2022	Acetamipryd 200 SL <i>Daphnia magna</i> , Acute Immobilisation Test Study code: W-11-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
	Czarnecka M.	2022	AMENDMENT NO. 1 TO THE FINAL REPORT Acetamipryd 200 SL <i>Daphnia magna</i> , Acute immobilisation test Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Poland Study code: W-11-22 GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/05 (filed as KCP 10.6.2/02)	Wróbel A.	2022	Acetamipryd 200 SL, Terrestrial Plant Test: Vegetative Vigour Test Study code: G-95-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
	Wróbel A.	2022	AMENDMENT NO. 1 TO THE FINAL REPORT Acetamipryd 200 SL Terrestrial Plant Test: Vegetative Vigour Test Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Poland Study code: G-95-21 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 5.1.2/06 (filed as KCP 10.6.2/01)	Wróbel A.	2022	Acetamipryd 200 SL, Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test Study code: G-96-21 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)	Wróbel A.	2022	Acetamipryd 200 SL Earthworm reproduction test (<i>Eisenia andrei</i>) Study code: G-93-21 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/08	Morsiani S.	2024	Effects of Acetamipryd 200 SL on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Chronic Oral Toxicity Test. Analytical Phase: Validation of an Analytical method and determination of content of Acetamiprid in the feeding solutions of honey bees new born workers (OECD 245) Report No. 23128-03R; 1032.11.SAG23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/09	Mautino G.	2024	Effects of Acetamipryd 200 SL on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure. Analytical Phase: Validation of an analytical method and determination of the content of Acetamiprid in the water stock solutions (OECD 239) Report No. 23128-04R; 1033.11.SAG23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/10	Mautino G.	2023	Predatory mites <i>Hypoaspis</i> (<i>Geolaelaps</i>) <i>aculeifer</i> reproduction test in soil with Acetamipryd 200 SL Analytical Phase:	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
			Validation of an analytical method and determination of content of Acetamiprid in soil samples (OECD 226) Report No. 23128-01R; 1039.1L.SAG23 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1.2/11 (filled as KCP 10.4.2.1/02)	Szlauer S.	2024	Collembolan (<i>Folsomia candida</i>) Reproduction Test in soil Study code: ETOX-2024-2 EcoTox Alliance Sp. z o. o. GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/12	Niewelt-Stasiak S.	2023	VALIDATION STUDY REPORT Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant) Validation Study No: VAL/10/2023 SGS GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/13	Niewelt-Stasiak S.	2023	VALIDATION STUDY REPORT Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in potato Validation Study No: VAL/11/2023 SGS GLP Unpublished	N	Pestila* ProAgri**
KCP 5.1.2/14	Niewelt-Stasiak S.	2023	VALIDATION STUDY REPORT Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in apple Validation Study No: VAL/12/2023 SGS	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
			GLP Unpublished		
KCP 5.1.2/15 and KCP 5.2/01	Lefebvre C.	2023	Determination of Acetamiprid Residues in Honey Following Application on Winter Oilseed Rape with Piorun 200 SL under semi field Conditions in Northern and Southern Europe in 2023 Report No. R C2051 ANADIAG GLP Unpublished	N	Pestila* ProAgri**
KCP 5.2/02	Niewelt-Stasiak S.	2024	INDEPENDENT LABORATORY VALIDATION - STUDY REPORT /draft/ Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in honey Report No. ILV/02/2023 SGS Polska Sp. z o. o. 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
KCP 5.2	Schwarz, T.	2008	Acetamiprid: Validation of an Enforcement Method for Plant Materials Study P/B1447G PTRL Europe Nippon-Soda Report No. RD-01937 GLP	N	Nippon Soda

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
			Upublished		
KCP 5.2	Weber, H.	2013	Validation of a Multiresidue Method (Fillion) with Modified Cleanup and Detection for the Determination of Acetamiprid in Potato Study No. S13-02134, Document ID RD-02603 Eurofins Agrosience Services GLP Unpublished	N	Nippon Soda
KCP 5.2	Giesau, A.	2012	Independent laboratory Validation of an Enforcement Method (“QuEChERS”) for the Determination of Residues of Acetamiprid in Crops using LC-MS/MS Study No. S12-02718, Document ID RD-02454 Eurofins Agrosience Services GLP Unpublished	N	Nippon Soda
KCP 5.2	Miya, K.	2010	Validation Study of the Analytical Method for the Determination of the Residues of Acetamiprid and Its Metabolite (IM-2-1) in Animal Commodities Report No. NCAS 10-144, Document ID RD-02080 Nisso Chemical Analysis Service Co., Japan GLP Unpublished	N	Nippon Soda
KCP 5.2	Knoch, E.	2010	Independent Laboratory Validation: Analytical Method for the Determination of the Residues of Acetamiprid and its Metabolite (IM-2-1) in Animal Commodities Report No. IF-10/01687868, Document ID RD-02156 SGS Institut Fresenius GmbH GLP Unpublished	N	Nippon Soda
KCP 5.2	Täufner, A. & Weber H.	2010	Validation of an Analytical Method for the Determination of Residues of Acetamiprid and Acetamiprid Soil Metabolite IM-1-5 in Calcareous Soil using LC-MS/MS Report No. S09-03287, Document ID RD-02062N Eurofins Dr. Specht, Germany GLP	N	Nippon Soda

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCP 5.2	Miya, K.	2007	Validation Study of the Confirmatory Method for the Determination of Acetamiprid in Water, Report No. NCAS 06-209, Document ID RD-01204 Nisso Chemical Analysis Service Co., Japan GLP Unpublished	N	Nippon Soda
KCP 5.2	Senciuc, M.	2014a	Independent Laboratory Validation (ILV) of a Residues Analytical Method for the Determination of Acetamiprid in Drinking Water Report No. P 3244 G, Document ID RD-02951 PTRL Europe GmbH, Germany GLP, Unpublished	N	Nippon Soda
KCP 5.2	Gieseau, A. & Weber, H.	2012	Validation of an Analytical Method for the Determination of Residues of Acetamiprid Metabolite IM-1-5 in Water using LC-MS/MS, Report No. S12-02719, Document ID RD-02604 Eurofins Agrosience Services, Germany, GLP, not published	N	Nippon Soda
KCP 5.2	Senciuc, M.	2014b	Independent Laboratory Validation (ILV) of a Residues Analytical Method for the Determination of Acetamiprid Metabolite IM-1-5 in Drinking Water Report No. P 3245 G, Document ID RD-02952 PTRL Europe GmbH, Germany GLP Unpublished	N	Nippon Soda
KCP 5.2	Beck, T., and Class, T.	2009	Acetamiprid: Development and Validation of an Analytical Method(s) for the Determination of Residues on Operator Exposure Dosimeters from Field Studies PTRL Europe, Germany Report No. P/B 1603 G, Document ID RD-01863 GLP not published	N	Nippon Soda

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	Senciuc, M.	2014c	Development and Validation of an Analytical Method for the Determination of Acetamiprid in Blood Report No. P3208 G, Document ID RD-02943 PTRL Europe, Germany GLP Unpublished	N	Nippon Soda

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 1 Detailed evaluation of submitted analytical methods

A 1.1 Analytical methods for acetamiprid

A 1.1.1 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 1.1.1.1 Description of analytical methods used in ecotoxicological studies

A 1.1.1.1.1 HPLC with DAD detection (in sucrose solution)

A 1.1.1.1.1.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/01 (filled as KCP 10.3.1.1.1/02)
Report	Acetamipryd 200 SL, Bumblebees (<i>Bombus</i> spp.), Acute Oral Toxicity Test, Fulczyk A., 2022, Report No. B-105-22
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method was developed for the determination of Acetamiprid 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5 µm C18(2) 100Å 250x4.6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of Acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of Acetamiprid into a volumetric flask 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 solution of Acetamiprid at concentration 100 µg/mL was prepared by dilution of the stock solution. The working solution of Acetamiprid at concentration 10 µg/mL was prepared by dilution of solution at concentration 100 µg/mL.

The working solutions containing of Acetamiprid at concentrations 10 and 100 µg/mL were prepared in acetonitrile for HPLC. Further dilutions were conducted with mixture acetonitrile for HPLC: deionized water (50:50; v/v).

Fortification samples

For validation experiments, 1g aliquot of untreated sucrose solution were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of sucrose solution an untreated (1g) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 5.0 mg acetamiprid/kg and ten times higher of LoQ 50.0 mg acetamiprid/kg. This was done to ensure the result fits within the range of the respective standard curve.

Sample preparation for the chromatographic analysis

1 g of sucrose solution was weighted into a volumetric flask with a capacity of 10 mL, 5 ml of deionized water was added and next the volume was made up to 10 mL with the acetonitrile for HPLC. The sample was diluted with mixture acetonitrile for HPLC : deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

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 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.

Linearity

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 2 mg acetamiprid/kg to 200 mg acetamiprid/kg in sucrose solution. The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery \pm relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid appropriate to level corresponding with LoQ and 10 x LoQ. Mean recoveries \pm relative standard deviation (RSD) 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.

Detected substance	Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	sucrose solution	5	5	99.8	0.5
		50	5	97.8	0.6

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the sucrose solution is from 0.5% to 0.6%.

The precision is 0.5% at level 5.0 mg acetamiprid/kg sucrose solution, 0.6% at level 50.0 mg acetamiprid/kg sucrose solution.

The RSD for method is $\leq 20\%$ 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 solvent i.e. mixture of acetonitrile for HPLC and deionized water (50:50; v/v) to standard preparing in control matrix at appropriate concentration. The matrix effects and concentrations are presented in table below.

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 exceeded $\pm 20\%$ in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	sucrose solution	0.5	1.3

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

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 $\leq 20\%$).

The LoQ is 5.0 mg acetamiprid/kg sucrose solution and equivalent to the calibration level at concentration 0.5 μg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 2.0 mg acetamiprid/kg sucrose solution and equivalent to the lowest calibration standard i.e. 0.2 μg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
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acetamiprid	5 mg/kg	2 mg/kg
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Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40, 47, 59, 63, 76 and 96 days of storage at cool temperature i.e. from 20C to 80C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 96 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.1. 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 1.1.1.1.2 HPLC with DAD detection (in 1% Triton water solution)

A 1.1.1.1.2.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/02 (filled as KCP 10.3.1.1.2/02)
Report	Acetamipryd 200 SL, Bumblebees (<i>Bombus</i> spp.), Acute Contact Toxicity Test, Fulczyk A., 2022, Report No. B-107-22
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method was developed for the determination of Acetamiprid in 1% Triton water 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5 µm C18(2) 100Å 250x4.6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of Acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of Acetamiprid into a volumetric flask 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 solution of Acetamiprid at concentration 100 µg/mL was prepared by dilution of the stock solution. The working solution of Acetamiprid at concentration 10 µg/mL was prepared by dilution of solution at concentration 100 µg/mL. The working solutions containing of Acetamiprid at concentrations 10 and 100 µg/mL were prepared in acetonitrile for HPLC. Further dilutions were conducted with mixture acetonitrile for HPLC: deionized water (50:50; v/v).

Fortification samples

For validation experiments, 1 mL aliquot of untreated 1% Triton water solution were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of 1% Triton water solution an untreated (1 mL) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 50.0 mg acetamiprid/L and ten times higher of LoQ 500.0 mg acetamiprid/L. This was done to ensure the result fits within the range of the respective standard curve.

Sample preparation for the chromatographic analysis

1 ml of 1% Triton water solution was taken and diluted in ratio 1 – 100 with mixture acetonitrile for HPLC : 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 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.

Linearity

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 20 mg acetamiprid/L to 2000 mg acetamiprid/L in 1% Triton water solution. The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery \pm relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid appropriate to level corresponding with LoQ and 10 x LoQ. Mean recoveries \pm relative standard deviation (RSD) 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.

Detected substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	1% Triton water solution	50	5	96.9	2.0
		500	5	95.7	3.5

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the 1% Triton water solution is from 2.0% to 3.5%. The precision is 2.0% at level 50.0 mg acetamiprid/L 1% Triton water solution, 3.5% at level 500.0 mg acetamiprid/L 1% Triton water solution. The RSD for method is $\leq 20\%$ 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 control matrix at appropriate concentration. The matrix effects and concentrations are presented in table below. 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 exceeded $\pm 20\%$ in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	1% Triton water solution	0.5	1.2

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

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 $\leq 20\%$).

The LoQ is 50.0 mg acetamiprid/L 1% Triton water solution and equivalent to the calibration level at concentration 0.5 μg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 20.0 mg acetamiprid/L 1% Triton water solution and equivalent to the lowest calibration standard i.e. 0.2 μg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	50 mg/L	20 mg/L

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12 and 40 days of storage at cool temperature i.e. from 20°C to 80°C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 40 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.1. 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% Triton water solution) for Bumblebees.

A 1.1.1.1.3 HPLC with DAD detection (in water)

A 1.1.1.1.3.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/03 (filed as KCP 10.2.1.3/01)
Report	Acetamipryd 200 SL, <i>Raphidocelis subcapitata</i> SAG 61.81 (formerly <i>Pseudokirchneriella subcapitata</i>), Growth inhibition test, Czarnecka, M., 2022, Report No. W-12-22 AMENDMENT NO. 1 TO THE FINAL REPORT Acetamipryd 200 SL <i>Raphidocelis subcapitata</i> SAG 61.81 (formerly <i>Pseudokirchneriella subcapitata</i>), Growth inhibition test, Czarnecka M; 2023; Report No. W-12-22
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method was developed for the determination of acetamiprid 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5µm C18 (2)100Å, l = 250 mm, φ = 4,6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of acetamiprid into a volumetric flask 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 solution of acetamiprid with a concentration of 100 µg/mL were prepared by dilution of the stock solution with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the solution at concentration 100 µg/mL with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 1 µg/mL were prepared by dilution of the solution at concentration 10 µg/mL with acetonitrile for HPLC. Calibration solutions containing of acetamiprid were prepared by dilution of the working solutions at concentration 1 µg/mL, 10 µg/mL and 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Fortification samples

For validation experiments, 5 mL aliquot of untreated water were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of water an untreated (5 mL) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 0.06 mg acetamiprid/L and ten times higher of LoQ 0.6 mg acetamiprid/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 water in a volume of 5 ml was diluted with acetonitrile for HPLC in ratio 1 -1. The sample was diluted mixture of acetonitrile for HPLC and deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

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.

Linearity

Calibration curve from 0.02 µg/mL to 2.0 µg/mL.

Working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.1, 0.20, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.02 µg/mL to 2 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.04 mg acetamiprid/L to 4.0 mg acetamiprid/L in water.

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

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.90299e-006	0.000613748	0.9999786

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

Calibration curve from 0.2 µg/mL to 20 µg/mL.

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.4 mg acetamiprid/L to 40 mg acetamiprid/L in water.

The equation of the calibration line is 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 was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid 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%.

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

Detected substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	water	0.06	5	112.8	0.3
		0.6	5	101.5	2.0

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in water is from 0.3% to 2.0%. The precision is 0.3% at level 0.06 mg acetamiprid/L water, 2.0% at level 0.6 mg acetamiprid/L water.

The RSD for method 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 solvent to standard preparing in control matrix at appropriate concentration. The matrix effect and concentration is presented in table below.

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 exceeded ± 20 % in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	water	0.03	7.5

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

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 $\leq 20\%$).

The LoQ is 0.06 mg acetamiprid/L water and equivalent to the calibration level at concentration 0.03 μg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 0.04 mg acetamiprid/L water and equivalent to the lowest calibration standard i.e. 0.02 μg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	0.06 mg/L	0.04 mg/L

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40, 47, 59 and 63 days of storage at cool temperature i.e. from 20C to 80C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 63 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830, Rev. 1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item matrix (water).

A 1.1.1.1.4 HPLC with DAD detection (in Elendt M7 medium)

A 1.1.1.1.4.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/04 (filed as KCP 10.2.1.2/01)

Report Acetamipryd 200 SL, *Daphnia magna*, Acute Immobilisation Test, Czarnecka, M., 2022, Report No. W-11-22

AMENDMENT NO. 1 TO THE FINAL REPORT

Acetamipryd 200 SL *Daphnia magna*, Acute immobilisation test, Czarnecka M; 2022; Report No. W-11-22

Guideline(s): SANTE/2020/12830, Rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Principle of the method

The analytical method was developed for the determination of acetamiprid 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) with DAD detection. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5µm C18 (2)100Å, l = 250 mm, φ = 4,6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of acetamiprid into a volumetric flask 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 solution of acetamiprid with a concentration of 100 µg/mL were prepared by dilution of the stock solution with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the solution at concentration 100 µg/mL with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 1 µg/mL were prepared by dilution of the solution at concentration 10 µg/mL with acetonitrile for HPLC. Calibration solutions containing of acetamiprid were prepared by dilution of the working solutions at concentration 1 µg/mL, 10 µg/mL and 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Fortification samples

For validation experiments, 5 mL aliquot of untreated Elendt M7 medium were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of Elendt M7 medium an untreated (5 mL) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 0.06 mg acetamiprid/L and ten times higher of LoQ 0.6 mg acetamiprid/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 Elendt M7 medium in a volume of 5 ml was diluted with acetonitrile for HPLC in ratio 1 -1. The sample was diluted mixture of acetonitrile for HPLC and deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

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 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.

Linearity

Calibration curve from 0.02 µg/mL to 2.0 µg/mL.

Working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.1, 0.20, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The

range of linearity of the analytical graph is from 0.02 µg/mL to 2 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.04 mg acetamiprid/L to 4.0 mg acetamiprid/L in Elendt M7 medium.

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

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.90299e-006	0.000613748	0.9999786

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

Calibration curve from 0.2 µg/mL to 20 µg/mL.

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.4 mg acetamiprid/L to 40 mg acetamiprid/L in Elendt M7 medium.

The equation of the calibration line is 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 was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid 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%.

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

Detected substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	Elendt M7 medium	0.06	5	108.3	0.6
		0.6	5	104.3	0.6

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in Elendt M7 medium is 0.6% for both fortification levels.

The precision is 0.6% at level 0.06 mg acetamiprid/L Elendt M7 medium, 0.6% at level 0.6 mg acetamiprid/L Elendt M7 medium.

The RSD for method 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 control matrix at

appropriate concentration. The matrix effect and concentration are presented in table below.
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 exceeded ± 20 % in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	Elendt M7 medium	0.03	2.4

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

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 $\leq 20\%$).

The LoQ is 0.06 mg acetamiprid/L Elendt M7 medium and equivalent to the calibration level at concentration 0.03 µg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 0.04 mg acetamiprid/L Elendt M7 medium and equivalent to the lowest calibration standard i.e. 0.02 µg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	0.06 mg/L	0.04 mg/L

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40 and 47 days of storage at cool temperature i.e. from 20C to 80C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 47 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830, Rev. 1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item matrix (Elendt M7 medium).

A 1.1.1.1.5 HPLC with DAD detection (in water)

A 1.1.1.1.5.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/05 (filed as KCP 10.6.2/02)

Report Acetamipryd 200 SL, Terrestrial Plant Test: Vegetative Vigour Test, Wróbel, A., 2022, Report No. G-95-21

AMENDMENT NO. 1 TO THE FINAL REPORT

Acetamipryd 200 SL Terrestrial Plant Test: Vegetative Vigour Test, Wróbel A., 2022, Report No. G-95-21

Guideline(s): SANTE/2020/12830, Rev. 1

Deviations: No

GLP: Yes

Acceptability: Yes

Principle of the method

The analytical method was developed for the determination of acetamiprid 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5µm C18 (2)100Å, l = 250 mm, φ = 4,6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of acetamiprid into a volumetric flask 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 solution of acetamiprid with a concentration of 100 µg/mL were prepared by dilution of the stock solution with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the solution at concentration 100 µg/mL with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 1 µg/mL were prepared by dilution of the solution at concentration 10 µg/mL with acetonitrile for HPLC. Calibration solutions containing of acetamiprid were prepared by dilution of the working solutions at concentration 1 µg/mL, 10 µg/mL and 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Fortification samples

For validation experiments, 5 mL aliquot of untreated water were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of water an untreated (5 mL) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 0.06 mg acetamiprid/L and ten times higher of LoQ 0.6 mg acetamiprid/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 water in a volume of 5 ml was diluted with acetonitrile for HPLC in ratio 1 -1. The sam-

ple was diluted mixture of acetonitrile for HPLC and deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

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.

Linearity

Calibration curve from 0.02 µg/mL to 2.0 µg/mL

Working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.1, 0.20, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.02 µg/mL to 2 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.04 mg acetamiprid/L to 4.0 mg acetamiprid/L in water.

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

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.90299e-006	0.000613748	0.9999786

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

Calibration curve from 0.2 µg/mL to 20 µg/mL

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.4 mg acetamiprid/L to 40 mg acetamiprid/L in water.

The equation of the calibration line is 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 was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery ± relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid 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%.

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

Detected substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
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acetamiprid	water	0.06	5	112.8	0.3
		0.6	5	101.5	2.0

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in water is from 0.3% to 2.0%.

The precision is 0.3% at level 0.06 mg acetamiprid/L water, 2.0% at level 0.6 mg acetamiprid/L water.

The RSD for method is $\leq 20\%$ 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 solvent to standard preparing in control matrix at appropriate concentration. The matrix effect and concentration are presented in table below.

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 exceeded $\pm 20\%$ in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	water	0.03	7.5

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

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 $\leq 20\%$).

The LoQ is 0.06 mg acetamiprid/L water and equivalent to the calibration level at concentration 0.03 μg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 0.04 mg acetamiprid/L water and equivalent to the lowest calibration standard i.e. 0.02 μg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	0.06 mg/L	0.04 mg/L

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40, 47 and 59 days of storage at cool temperature i.e. from 20C to 80C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 59 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830, Rev. 1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item matrix (water).

A 1.1.1.1.6 HPLC with DAD detection (in water)

A 1.1.1.1.6.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/06 (filed as KCP 10.6.2/01)
Report	Acetamipryd 200 SL, Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, Wróbel, A., 2022, Report No. G-96-21
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method was developed for the determination of acetamiprid 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5µm C18 (2)100Å, l = 250 mm, φ = 4,6 mm
Wavelength:	245 nm
Injection volume:	20 µL
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of acetamiprid into a volumetric flask 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 solution of acetamiprid with a concentration of 100 µg/mL were prepared by dilution of the stock solution with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the solution at concentration 100 µg/mL with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 1 µg/mL were prepared by dilution of the solution at concentration 10 µg/mL with acetonitrile for HPLC. Calibration solutions containing of acetamiprid were prepared by dilution of the working solutions at concentration 1 µg/mL, 10 µg/mL and 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Fortification samples

For validation experiments, 5 mL aliquot of untreated water were spiked with appropriate volumes of

common fortification solutions of acetamiprid. Sample of water an untreated (5 mL) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 0.06 mg acetamiprid/L and ten times higher of LoQ 0.6 mg acetamiprid/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 water in a volume of 5 ml was diluted with acetonitrile for HPLC in ratio 1 -1. The sample was diluted mixture of acetonitrile for HPLC and deionized water (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

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.

Linearity

Calibration curve from 0.02 µg/mL to 2.0 µg/mL

Working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.1, 0.20, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.02 µg/mL to 2 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.04 mg acetamiprid/L to 4.0 mg acetamiprid/L in water.

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

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.90299e-006	0.000613748	0.9999786

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

Calibration curve from 0.2 µg/mL to 20 µg/mL

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.4 mg acetamiprid/L to 40 mg acetamiprid/L in water.

The equation of the calibration line is 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 was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery \pm relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid appropriate to level corresponding with LoQ and 10 x LoQ. Mean recoveries \pm relative standard deviation (RSD) 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.

Detected substance	Matrix	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	water	0.06	5	112.8	0.3
		0.6	5	101.5	2.0

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in water is from 0.3% to 2.0%. The precision is 0.3% at level 0.06 mg acetamiprid/L water, 2.0% at level 0.6 mg acetamiprid/L water. The RSD for method is \leq 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 solvent to standard preparing in control matrix at appropriate concentration. The matrix effect and concentration are presented in table below. 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 exceeded \pm 20 % in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	water	0.03	7.5

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

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 \leq 20%).

The LoQ is 0.06 mg acetamiprid/L water and equivalent to the calibration level at concentration 0.03 μ g acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a sample.

The LoD is 0.04 mg acetamiprid/L water and equivalent to the lowest calibration standard i.e. 0.02 μ g acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	0.06 mg/L	0.04 mg/L

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40, 47 and 59 days of storage at cool temperature i.e. from 2°C to 8°C. Compared to the mean recoveries measured at 0 day, significant decline was not ob-

served after 59 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830, Rev. 1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item matrix (water).

A 1.1.1.1.7 HPLC with DAD detection (in artificial soil)

A 1.1.1.1.7.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/07 (filled as KCP 10.4.1.1/01)
Report	Acetamipryd 200 SL, Earthworm reproduction test (<i>Eisenia andrei</i>), Wróbel A., 2022, Report No. G-93-21
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Principle of the method

The analytical method was developed for the determination of acetamiprid 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.

Materials and methods

Chromatographic system:	High Performance Liquid Chromatography (HPLC)
Chromatograph:	Shimadzu, Prominence- <i>i</i> (Shimadzu Corporation Japan)
Analytical column:	Luna 5 µm C18(2) 100Å 250x4.6 mm
Wavelength:	245 nm
Injection volume:	20 µl
Oven temperature:	35°C
Mobile phase:	acetonitrile for HPLC : ortho-phosphoric acid solution 0.05% (40 : 60, v/v)
Flow rate:	0.9 mL/min
Detection System:	Diode Array Detector

Working and fortifications solutions

Stock and standard solutions

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standard of acetamiprid into a volumetric flask 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 solution of acetamiprid with a concentration of 100 µg/mL were prepared by dilution of the stock solution with acetonitrile for HPLC. The working solution of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the solution at concentration 100 µg/mL with acetonitrile for HPLC. The working

solution of acetamiprid with a concentration of 1 µg/mL were prepared by dilution of the solution at concentration 10 µg/mL with acetonitrile for HPLC. Calibration solutions containing of acetamiprid were prepared by dilution of the working solutions at concentration 1 µg/mL, 10 µg/mL and 100 µg/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v).

Fortification samples

For validation experiments, 10 g aliquot of untreated artificial soil were spiked with appropriate volumes of common fortification solutions of acetamiprid. Sample of artificial soil an untreated (10 g) was spiked with the solution of acetamiprid to achieve fortification levels at the limit of quantification i.e. 0.05 mg acetamiprid/kg and ten times higher of LoQ 0.5 mg acetamiprid/kg. This was done to ensure the result fits within the range of the respective standard curve.

Sample preparation for the chromatographic analysis

First, 15 mL of ethyl acetate, was added to 10 g of artificial soil sample and shaken for 5 minutes. The organic phases were centrifuged and filtered through anhydrous sodium sulphate (VI). The extraction was repeated. The extracts were evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in mixture of acetonitrile : 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 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.

Linearity

Calibration curve from 0.02 mg/L to 2.0 mg/L

Working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.1, 0.20, 0.5, 1.0 and 2.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.02 µg/mL to 2 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.02 mg acetamiprid/kg to 2.0 mg acetamiprid/kg in artificial soil. The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.90299e-006	0.000613748	0.9999786

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

Calibration curve from 0.2 mg/L to 20 mg/L

Working solutions of acetamiprid at the concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of linearity of the analytical graph is from 0.2 µg/mL to 20 µg/mL. The range of calibration curve of acetamiprid is equivalent to range from 0.2 mg acetamiprid/kg to 20 mg acetamiprid/kg in artificial soil. The equations of the calibration line were presented as the linear equation; $y = ax + b$ (a – slope, b - intercept). The linear coefficient r^2 must be higher than 0.99. Range of the linear was given in µg/mL equivalent to mg/L.

lent to mg/L.

Analyte	Slope	Intercept	Coefficient
acetamiprid	8.76839e-006	0.0106739	0.9997348

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

Accuracy

The accuracy of the method is reported as mean recovery \pm relative standard deviation. Recovery data was reported for 2 fortification levels of acetamiprid appropriate to level corresponding with LoQ and 10 x LoQ. Mean recoveries \pm relative standard deviation (RSD) 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.

Detected substance	Matrix	Fortification Level [mg/kg]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	Artificial soil	0.05	5	89.0	1.8
		0.5	5	79.1	0.2

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in artificial soil is from 0.2% to 1.8%.

The precision is 1.8% at level 0.05 mg acetamiprid/kg artificial soil, 0.2% at level 0.5 mg acetamiprid/kg artificial soil.

The RSD for method is $\leq 20\%$ 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 control matrix at appropriate concentration. The matrix effect and concentration is presented in table below.

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 exceeded $\pm 20\%$ in of the method.

Detected substance	Matrix	Concentration [mg/L]	matrix effect [%]
acetamiprid	Artificial soil	0.05	0.8

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

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 $\leq 20\%$).

The LoQ is 0.05 mg acetamiprid/kg artificial soil and equivalent to the calibration level at concentration 0.05 μg acetamiprid/mL.

The limit of detection (LoD) is defined as the lowest detectable concentration or amount of an analyte in a

sample.

The LoD is 0.02 mg acetamiprid/kg artificial soil and equivalent to the lowest calibration standard i.e. 0.02 µg acetamiprid/mL.

Detected substance	LOQ (limit of quantification)	LOD (limit of detection)
acetamiprid	0.05 mg/kg	0.02 mg/kg

Stock solution stability

The stability of stock solution was tested at concentrations 1 mg/mL i.e. 1000 mg acetamiprid/L. The results for stability were obtained after 0, 1, 2, 12, 40, 47, 59, 63, 76 and 96 days of storage at cool temperature i.e. from 20C to 80C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 96 days. The mean recovery for each of the solutions do not differ by more than 10%.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the test item in matrix (artificial soil).

A 1.1.1.1.8 HPLC tandem LC-MS/MS (in sucrose solution)

A 1.1.1.1.8.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/08

Report Effects of Acetamipryd 200 SL on Honeybees (*Apis mellifera* L.) in the laboratory – Chronic Oral Toxicity Test.

Analytical Phase:

Validation of an Analytical method and determination of content of Acetamiprid in the feeding solutions of honey bees new born workers (OECD 245)

Morsiani S., 2024, Report No. 23128-03R; 1032.1I.SAG23

Guideline(s): SANTE/2020/12830 rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The analytical method for acetamiprid in sucrose solution matrix was fully validated according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).

Acetamiprid was extracted from the sucrose solution matrix using acetonitrile, after addition of an opportune amount of water. After QuEChERS salts addition, acetonitrile phase was separated from the aqueous phase. An aliquot of the acetonitrile extract was further diluted and was analysed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Two transitions m/z 223 > 126 and m/z 223 > 90 were acquired: the first transition (target) for quantifica-

tion purpose, the second transition (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: HPLC Series 1200 Agilent
- MS/MS detector System: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases:
 - Solvent A: Water (milliQ), 0.1% formic acid, 5mM ammonium formate
 - Solvent B: Methanol (UHPLC-MS), 0.1% formic acid, 5 mM ammonium formate
- Pump:
 - 0 min: A 50 % - B 50 %
 - 4 min: A 50 % - B 50 %
- Flow rate: 0.3 mL/min
- Column Temperature: 35 °C
- Injection volume: 2 µL
- Retention time: Acetamiprid: ~1.6 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
 - Temperature (TEM): 500 °C
 - Curtain gas (CUR): 20 psi
 - Collision gas (CAD): 5 psi
 - Ion Spray Voltage (IS): 5500 V
 - Gas 1: 45 psi
 - Gas 2: 45 psi
 - DP: 42.1
 - EP: 7.00
- Transitions:
 - Acetamiprid-1_T (223/126) CE 30, CXP 2.5, dwell time 200 msec
 - Acetamiprid-2_Q (223/90): CE 46, CXP 3.0, dwell time 200 msec

Stock and working reference item solutions

20.1 mg of the reference item were weighed into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at acetamiprid concentration 1002.8 µg/mL; stored in freezer at temperature ≤ - 18 °C).

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ - 18 °C in the dark in analytical phase 23128-02R.

By dilution of the reference item stock solution described above with acetonitrile, intermediate reference item working solutions were prepared, as reported in the table below.

Acetamiprid reference item parent solution concentration (µg/mL)	Taken Volume (mL)	Final Volume (mL)	Acetamiprid reference item working solution concentration (µg/mL)
1002.8	0.2	20	10.028
10.028	0.5	10	501.40

These intermediate acetamiprid standard solutions were freshly prepared on the analysis day and used to prepare calibration standard solutions.

Stock and working test item solutions

1. 173.5 mg of test item were weighed into a 5 mL volumetric flask and made up to volume with demineralised water: test item stock solution at acetamiprid concentration 5982 µg/mL (197.2 g/L acetamiprid active ingredient content and 1.144 g/mL density taken into account). This stock solution was used to spike sucrose solution recovery samples at 2nd level and was prepared freshly before use.
2. 1.0 mL of the stock solution described at point 1 was transferred into a 25 mL volumetric flask and made up to volume with demineralized water to obtain a test item working solution at acet-

amiprid concentration 239.3 µg/mL. This working solution was used to spike sucrose solution recovery samples at LOQ level and was prepared freshly before use.

Fortification procedure

The fortification procedure is described in the following table.

Matrix	Matrix amount	Fortification level (mg/kg)	Spiking solution concentration (µg/mL)	Spiking solution volume
Sucrose solutions	2.0 g	10 mg/kg (LOQ) 299 mg/kg (2nd level)	239.3 5982	0.085 0.100

Analytical procedure for sample extracts preparation: matrix sucrose solution

The homogenised sample (2.00 ± 0.05) was weighted into a 50 mL centrifuge tube; recovery samples were fortified at this point.

Then 8 mL of demineralised water were added and the sample was manually shaken for a few seconds.

In recovery trials, the fortification volume comprised the water volume.

Then 10 mL of acetonitrile were added. The tube was shaken vigorously by hand for 1 minute. After this step, the content of a sachet of Quechers EN method was added to the sample.

The tube was shaken vigorously by hand for 1 minute and then centrifuged at 4000 rpm for five minutes.

The extract was diluted 1:200 with acetonitrile (final volume 2000 mL).

Filter the final sample extract by PTFE filter, porosity 0.22 µm.

Sample extracts with analyte concentration exceeding 40 ng/mL were opportunely diluted with solvent acetonitrile in order to fall within the $\pm 20\%$ of the calibration range.

Validation

Linearity

The instrument was calibrated by reading six acetamiprid solvent standard solutions in the concentration range 1.0 – 50 ng/mL (injected before and after the samples, averaging the responses), corresponding to 1.0 – 50 mg/kg in undiluted samples.

The correlation coefficients of the weighed linear (1/x) multipoint external solvent standard calibration curves was found ≥ 0.9975 in the analytical sequence performed.

The linearity range comprised the concentration range from 20% of the LOQ to at least 20% above the highest measured concentration.

The suitability of the calibration lines for the matrix sucrose solution was assessed using the residuals d_i that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - y_{yi}$$

where:

y_i is the measured value i ;

y_{yi} is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if d_i were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.

The linearity range comprised the concentration range of the samples $\pm 20\%$.

Blank and selectivity

The analysis of the untreated (blank) sample was performed in duplicate: no significant interference exceeding 30 % of the limit of quantification was found at the retention time of acetamiprid for the monitored transitions.

Therefore, acetamiprid can be regarded as not detectable in untreated sucrose solution samples used in fortification trials.

The retention time of the reference item matched the retention time of the analyte in extracts from forti-

fied samples.

Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of acetamiprid in sucrose solution matrix, without significant interferences above 30 % of LOQ.

Specificity

Acetamiprid was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.

The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 70-140 m/z in the present study.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.

The recoveries were performed by fortifying the untreated blank samples at two levels.

The LOQ level was set at acetamiprid concentration of 10 mg/kg (lower than the minimum expected acetamiprid content in the treated samples) while the second level was set at 299 mg/kg (higher than the maximum expected concentration in the treated samples), in order to cover all the range of acetamiprid concentrations in the analytical samples. Five replicated analyses were carried out for each fortification level.

The background content in the control sample used in fortification experiments was not detectable.

In these recovery samples the acetamiprid content was determined as reported in table below.

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
10 (a) (LOQ)	99.0 104.2 101.6 102.0 100.9	101.5	1.9	101.6 \pm 4.1
299 (b) (2 nd level)	105.4 110.2 97.4 96.0 98.8	101.6	5.9	

(a) 59 mg/kg as test item

(b) 1735 mg/kg as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy

The accuracy of the analysis method for acetamiprid in sucrose solution, defined as mean recovery \pm relative standard deviation, is 101.6 \pm 4.1 for 10 fortified samples analysed.

Repeatability

The repeatability, defined as the % RSD (Relative Standard Deviation) at each fortification level, and the overall RSD of sucrose solution matrix are reported in table below.

Fortification level (mg/kg)	RSD (%) (n=5)	Overall RSD % (n=10)
10 (a) (LOQ)	1.9	4.1
299 (b) (2 nd level)	5.9	

- (a) 59 mg/kg as test item
(b) 1735 mg/kg as test item

For each fortification level, the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.

The LOQ for acetamiprid in sucrose solution matrix was assessed in this study at 10 mg/kg.

Limit of detection (LOD)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.

For the analysis of acetamiprid in sucrose solution matrix the LOD was 1.0 mg/kg.

This value was calculated from the acetamiprid concentration corresponding to the lowest calibration point, which is < 30% of the LOQ.

Matrix effects

To check possible signal enhancement or suppression effects in the LC-MS/MS analysis, the response of a matrix-matched standard at LOQ level was compared with the response of the same standard solution concentration in solvent. The solvent standard was injected before and after the corresponding matrix matched standard and the response of the two injections averaged. The results are summarized in the following table.

Matrix	Transition	Mean Matrix response Over solvent response %	Matrix effects %
Sucrose solutions	223/126 (Primary)	85	-15
	223/90 (Confirmatory)	83	-17

No significant matrix effect (i.e., exceeding ± 20 %) was found. For the quantification of sucrose solution samples, solvent calibration standards were used.

Confirmation

The confirmation of the analyte's identity was achieved simultaneously to the primary detection by the acquisition of the additional transition 223/90.

The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
10 (a) (LOQ)	99.0 104.1 101.2 105.6 103.5	102.7	2.5	102.5 \pm 4.4
299 (b) (2 nd level)	106.2 111.5 96.4 98.1 99.7	102.4	6.2	

- (a) 59 mg/kg as test item
(b) 1735 mg/kg as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830

rev.2.

Stability of final extracts and reference item solutions

During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.

The final extracts were analysed within 24 hours form extraction therefore additional stability testing was not required.

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ -18 °C in the dark in study 23128-02R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ -18 °C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.2.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.2. The data presented above confirm that the validated analytical method provides specific, reliable, accurate and precise procedures for the determination of acetamiprid active ingredient in sucrose solution samples in the range 10 - 299 mg/kg (corresponding to 59 - 1735 mg/kg as test item).

A 1.1.1.1.9 HPLC tandem LC-MS/MS (in water)

A 1.1.1.1.9.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference:	KCP 5.1.2/09
Report	Effects of Acetamipryd 200 SL on Honeybees (<i>Apis mellifera</i> L.) in the laboratory – Larval Toxicity Test Following Repeated Exposure. Analytical Phase: Validation of an analytical method and determination of the content of Acetamiprid in the water stock solutions (OECD 239) Mautino G., 2024, Report No. 23128-04R; 1033.1L.SAG23
Guideline(s):	SANTE/2020/12830 rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for acetamiprid in water matrix was fully validated according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).

Acetamiprid was determined in water solutions after dilution with acetonitrile/ water 50:50 v/v and the final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Two transitions m/z 223 > 126 and m/z 223 > 90 were acquired: the first transition (target) for quantification purpose, the second transition (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: HPLC Series 1200 Agilent
- MS/MS detector System: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases:
 - Solvent A: Water (milliQ), 0.1% formic acid, 5mM ammonium formate
 - Solvent B: Methanol (UHPLC-MS), 0.1% formic acid, 5 mM ammonium formate
- Pump:
 - 0 min: A 50 % - B 50 %
 - 4 min: A 50 % - B 50 %
- Flow rate: 0.3 mL/min
- Column Temperature: 35 °C
- Injection volume: 2 µL
- Retention time: Acetamiprid: ~1.6 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
 - Temperature (TEM): 500 °C
 - Curtain gas (CUR): 20 psi
 - Collision gas (CAD): 5 psi
 - Ion Spray Voltage (IS): 5500 V
 - Gas 1: 45 psi
 - Gas 2: 45 psi
 - DP: 42.1
 - EP: 7.00
- Transitions:
 - Acetamiprid-1_T (223/126) CE 30 , CXP 2.5, dwell time 200 msec
 - Acetamiprid-2_Q (223/90): CE 46 , CXP 3.0, dwell time 200 msec

Stock and working reference item solutions

20.1 mg of the reference item were weighed into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at acetamiprid concentration 1002.8 µg/mL; stored in freezer at temperature ≤ - 18 °C).

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ - 18 °C in the dark in analytical phase 23128-02R.

By dilution of the reference item stock solution described above with acetonitrile, intermediate reference item working solutions were prepared, as reported in the table below.

Acetamiprid reference item parent solution concentration (µg/mL)	Taken Volume (mL)	Final Volume (mL)	Acetamiprid reference item working solution concentration (µg/mL)
1002.8	0.2	20	10.028
10.028	0.5	10	501.40

These intermediate acetamiprid standard solutions were freshly prepared on the analysis day and used to prepare calibration standard solutions.

Stock and working test item solutions (fortification procedure)

- 214.9 mg of test item were weighed into a 20 mL volumetric flask and made up to volume with demineralised water: test item stock solution at acetamiprid concentration 1852 µg/mL (197.2 g/L acetamiprid active ingredient content and 1.144 g/mL density taken into account). This stock solution was used as recovery solution at the second fortification level and was prepared freshly before use.
- 0.7 mL of the stock solution described at point 1 was transferred into a 25 mL volumetric flask and made up to volume with demineralized water to obtain a test item working solution at acetamiprid concentration 51.87 µg/mL. This test item working solution was an intermediate used to prepare water recovery samples at LOQ level and was prepared freshly before use.

3. 0.2 mL of the stock solution described at point 2 was transferred into a 20 mL volumetric flask and made up to volume with demineralized water to obtain a test item working solution at acetamiprid concentration 0.5187 µg/mL. This test item working solution was used as water recovery samples at LOQ level and was prepared freshly before use

The fortification levels of recovery samples covered all the range of acetamiprid concentrations in the analytical samples of study 1033.11.SAG23.

Analytical procedure for sample extracts preparation: matrix water

The sample was let to warm up to room temperature, then sonicated for 5 minutes, shaken by a vortex for a further minute and immediately 1 mL was taken and transferred into a 10 mL volumetric flask, making up to volume with acetonitrile/water 50:50 v/v solvent mixture.

The sample was then further diluted 1:10 with the same acetonitrile/water solvent mixture (final extraction volume 100 mL).

Recovery samples were prepared in water using the test item.

Sample extracts with analyte concentration exceeding 48 ng/mL were opportunely diluted with acetonitrile/water solvent mixture in order to fall within the $\pm 20\%$ of the calibration range.

The extract was finally analysed by HPLC-MS/MS method.

Quantification was performed using solvent calibration standards in the concentration range 1 – 60 ng/mL.

Recovery samples at second level were diluted 1:500 with acetonitrile/water solvent mixture in order to obtain final extract concentrations falling within $\pm 20\%$ of the calibration range.

Validation

Linearity

The instrument was calibrated by reading six acetamiprid solvent standard solutions in the concentration range 1.0 – 60 ng/mL (injected before and after the samples, averaging the responses), corresponding to 0.00010 - 0.0060 g/L in undiluted samples.

The correlation coefficients of the weighed linear (1/x) multipoint external solvent standard calibration curves was found ≥ 0.9997 in the analytical sequence performed.

The linearity range comprised the concentration range from 20% of the LOQ to at least 20% above the highest measured concentration.

The suitability of the calibration lines for the water matrix was assessed using the residuals d_i that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - \hat{y}_i$$

where:

y_i is the measured value i ;

\hat{y}_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if d_i were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.

The linearity range comprised the concentration range of the samples $\pm 20\%$.

Blank and selectivity

The analysis of the untreated (blank) sample was performed in duplicate: no significant interference exceeding 30 % of the limit of quantification was found at the retention time of acetamiprid for the monitored transitions.

Therefore, acetamiprid can be regarded as not detectable in untreated sucrose solution samples used in fortification trials.

The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.

Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of

acetamiprid in water matrix, without significant interferences above 30 % of LOQ.

Specificity

Acetamiprid was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.

The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 70-140 m/z in the present study.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.

The recoveries were performed by fortifying the untreated blank samples at two levels.

The LOQ level was set at acetamiprid concentration of 0.00052 g/L (0.0030 g/L as test item), lower than the minimum found acetamiprid content in samples, while the second level was set at 1.9 g/L (11 g/L as test item), higher than the maximum expected concentration in the samples, in order to cover with the method validation all the range of acetamiprid concentrations in the analytical samples. Five replicated analyses were carried out for each fortification level.

The background content in the control sample used in fortification experiments was not detectable.

In these recovery samples the acetamiprid content was determined as reported in table below.

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.00052 (a) (LOQ)	98.3 101.2 100.3 98.9 102.2	100.2	1.6	101.3 \pm 2.7
1.9 g/L (b) (2 nd level)	99.0 107.6 103.1 100.1 102.7	102.5	3.3	

(a) 0.0030 g/L as test item

(b) 11 g/L as test item

For each fortification level the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy

The accuracy of the analysis method for acetamiprid in water, defined as mean recovery \pm relative standard deviation, is 101.3 \pm 2.7 for 10 fortified samples analysed.

Repeatability

The repeatability, defined as the % RSD (Relative Standard Deviation) at each fortification level, and the overall RSD of water matrix are reported in table below.

Fortification level (mg/kg)	RSD (%) (n=5)	Overall RSD % (n=10)
0.00052 (a) (LOQ)	1.6	2.7
1.9 g/L (b) (2 nd level)	3.3	

(a) 0.0030 g/L as test item

(b) 11 g/L as test item

For each fortification level, the mean recovery and the precision (RSD, relative standard deviation) are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.

The LOQ for acetamiprid in water matrix was assessed in this study at 0.00052 g/L.

Limit of detection (LOD)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.

For the analysis of acetamiprid in water matrix the LOD was 0.00010 g/L.

This value was calculated from the acetamiprid concentration corresponding to the lowest calibration point, which is < 30% of the LOQ.

Matrix effects

To check possible signal enhancement or suppression effects in the LC-MS/MS analysis, the response of a matrix-matched standard at LOQ level was compared with the response of the same standard solution concentration in solvent. The solvent standard was injected before and after the corresponding matrix matched standard and the response of the two injections averaged. The results are summarized in the following table.

Matrix	Transition	Mean Matrix response Over solvent response %	Matrix effects %
Water	223/126 (Primary)	98	-2
	223/90 (Confirmatory)	98	-2

No significant matrix effect (i.e., exceeding ± 20 %) was found. For the quantification of water samples, solvent calibration standards were used.

Confirmation

The confirmation of the analyte's identity was achieved simultaneously to the primary detection by the acquisition of the additional transition 223/90.

The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.00052 (a) (LOQ)	102.5 103.0 101.0 99.3 101.4	101.4	1.4	101.9 \pm 2.1
1.9 g/L (b) (2 nd level)	99.1 106.6 102.8 101.4 101.4	102.3	2.7	

(a) 0.0030 g/L as test item

(b) 11 g/L as test item

Also, for the confirmatory transition, the mean recovery and the precision (RSD, relative standard deviation) for each fortification level are in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability of final extracts and reference item solutions

During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.

The final extracts were analysed within 24 hours form extraction therefore additional stability testing was not required.

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ -18 °C in the dark in study 23128-02R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ -18 °C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.2.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.2. The data presented in this report confirm that the validated analytical method provides specific, reliable, accurate and precise procedures for the determination of acetamiprid active ingredient in water samples in the range 0.00052 - 1.9 g/L (corresponding to 0.0030 - 11 g/L as test item).

A 1.1.1.1.10 HPLC tandem LC-MS/MS (in soil)

A 1.1.1.1.10.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/10

Report: Predatory mites *Hypoaspis (Geolaelaps) aculeifer* reproduction test in soil with Acetamipryd 200 SL

Analytical Phase:

Validation of an analytical method and determination of content of Acetamiprid in soil samples (OECD 226)

Mautino G., 2023, Report No. 23128-01R; 1039.11.SAG23

Guideline(s): SANTE/2020/12830 rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The analytical method for acetamiprid in soil was fully validated according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).

Acetamiprid was extracted from soil samples with acetonitrile, after an addition of an opportune amount of water. After QuEChERS salts addition, the acetonitrile phase was separated from the aqueous phase.

The final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).

Two transitions m/z 223 > 126 and m/z 223 > 90 were acquired: the first transition (target) for quantification purpose, the second transition (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: HPLC Series 1200 Agilent
- MS/MS detector System: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases:
 - Solvent A: Water (milliQ), 0.1% formic acid, 5mM ammonium formate
 - Solvent B: Methanol (UHPLC-MS), 0.1% formic acid, 5 mM ammonium formate
- Pump:
 - 0 min: A 50 % - B 50 %
 - 4 min: A 50 % - B 50 %
- Flow rate: 0.3 mL/min
- Column Temperature: 35 °C
- Injection volume: 2 µL
- Retention time: Acetamiprid: ~1.5 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)
 - Temperature (TEM): 500 °C
 - Curtain gas (CUR): 20 psi
 - Collision gas (CAD): 5 psi
 - Ion Spray Voltage (IS): 5500 V
 - Gas 1: 45 psi
 - Gas 2: 45 psi
 - DP: 42.1
 - EP: 7.00
- Transitions:
 - Acetamiprid-1_T (223/126) CE 30, CXP 2.5, dwell time 200 msec
 - Acetamiprid-2_Q (223/90): CE 46, CXP 3.0, dwell time 200 msec

Stock and working reference item solutions

20.0 mg of the reference item were weighed into a 20 mL volumetric flask and brought to volume with acetonitrile (stock solution at acetamiprid concentration 1002.8 µg/mL; stored in freezer at temperature ≤ - 18 °C).

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ - 18 °C in the dark in analytical phase 23128-02R.

By dilution of the reference item stock solution described above with acetonitrile, intermediate reference item working solutions were prepared, as reported in the table below (nominal values).

Acetamiprid reference item parent solution concentration (µg/mL)	Taken Volume (mL)	Final Volume (mL)	Acetamiprid reference item working solution concentration (µg/mL)	Solution type
997.8	0.2	20	9.978	Intermediate
9.978	0.04	10	39.912	Intermediate
39.912	0.1	1	3.9912	Matrix effects check

The acetamiprid reference item solvent working solutions were freshly prepared on the analysis day.

Test item solutions for fortification

1248.5 mg of the test item were weighed into a 5 mL volumetric flask and diluted to volume with demineralised water obtaining a test item stock solution containing 43048 µg/mL of acetamiprid used to spike the untreated matrix at the second fortification level.

By dilution of the test item stock solution (0.1 mL in 100 mL of demineralised water and then 1 mL in 10 mL of demineralized water) a solution containing 4.305 µg/mL of acetamiprid active ingredient was obtained: this test item working solution was used to spike the untreated matrix at LOQ fortification level.

Fortification procedure

The fortification procedure is described in the following table:

Matrix	Matrix weight (*) (g)	Fortification level (mg/kg) (*)	Spiking solution concentration (µg/mL)	Spiking solution Volume (mL)
Soil	4.3 g	0.05 mg/kg (LOQ) 350 mg/kg (2nd level)	4.305 43048	0.05 0.035

(*) dry soil weight (soil wet weight 5 g; dry matter ratio 0.86)

Analytical procedure for soil samples analysis

The sample was let to warm up to room temperature.

The homogenised sample (5 ± 0.1 g) was weighted into a 50 mL centrifuge tube: recovery samples were fortified at this point.

Then 8 mL of demineralised water were added and the sample was shaken for a few seconds using a vortex shaker to homogenise.

Then 10 mL of acetonitrile were added, the sample was manually shaken for a minute. After this step, the content of a sachet of QuEChERS was added to the sample.

The tube was shaken vigorously by hand for 1 minute and then centrifuged at 4000 rpm for five minutes.

Then 1 mL of the supernatant layer was transferred into a 5 mL volumetric flask and made up to volume with demineralised water (final extract volume 50 mL)

The final extract was filtered with PTFE filter, porosity 0.20 µm and analysed by LC-MS/MS method.

Quantification was conducted using an external standard calibration curve obtained by linear regression of matrix matched calibration standards injected throughout the run in the range 1 – 50 ng/mL.

Sample extracts with analyte concentration exceeding 40 ng/mL were opportunely diluted with the untreated blank extract in order to fall within the ± 20 % of the calibration range.

Validation

Linearity

The linearity range for acetamiprid was found between 1 - 50 ng/mL corresponding to 0.0116 to 0.580 mg/kg of acetamiprid in dry soil sample. The correlation coefficient of the weighed linear (1/x) multipoint external matrix matched standard calibration curves was found ≥ 0.999 for both ion transitions in all the analytical sequences performed.

The linearity range comprised the concentration range from 30 % of the LOQ to at least 20 % above the highest measured concentration.

The suitability of the calibration lines for the matrix sucrose solution was assessed using the residuals d_i that describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - yy_i$$

where:

y_i is the measured value i ;

yy_i is the estimated value which corresponds to y_i and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if d_i were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plotting the residuals vs the concentration.

Blank and selectivity

The analysis of the untreated (blank) sample was performed in duplicate: no significant interference exceeding 30 % of the limit of quantification was found at the retention time of acetamiprid for the monitored transitions.

Therefore, acetamiprid can be regarded as not detectable in untreated sucrose solution samples used in fortification trials (< 30 % of LOQ).

The retention time of the reference item matched the retention time of the analyte in extracts from fortified samples.

Based on the analysis of the blank matrix, the method was confirmed to be selective for the analysis of acetamiprid in sucrose solution matrix, without significant interferences above 30 % of LOQ.

Specificity

Acetamiprid was analysed by MS/MS highly specific detection system; two transitions were simultaneously acquired: one transition, the target one, for quantification and one transition, the qualifier one, for confirmation.

The mass spectrum (product ion chromatogram) of the analyte was acquired in the range 70-140 m/z in the present study.

Recoveries

The analytical method was validated by recovery trials: a known quantity of the test item was added to the control sample and the percentage recovery calculated.

The recoveries were performed by fortifying the untreated blank samples at two levels.

The LOQ level was set at acetamiprid concentration of 0.05 mg/kg dry weight (lower than the minimum found acetamiprid content in samples, while the second level was at 350 mg/kg dry weight (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of acetamiprid concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.

The background content in the control sample used in fortification experiments was not detectable.

In these recovery samples the acetamiprid content was determined as reported in table below.

Fortification level (mg/kg DW)	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery \pm RSD %
0.05 (LOQ)	104.1 104.0 98.8 96.9 100.6	100.9	3.1	98.0 \pm 4.3
350 (II level)	92.5 95.3 93.1 94.7 100.1	95.1	3.2	

DW = Dry Weight

For each fortification level the mean recovery was in the range 70 – 120 % and the precision (RSD, relative standard deviation) \leq 20 %, in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy

The accuracy of the analysis method for acetamiprid in soil, defined as mean recovery \pm relative standard deviation, is 98.0 \pm 4.3.

Repeatability

The repeatability, defined as the % RSD (Relative Standard Deviation) at each fortification level, and the overall RSD is reported in the following table.

Fortification level (mg/kg _{DW})	RSD (%) (n=5)	Overall RSD % (n=10)
0.05 (LOQ)	3.1	4.3
350 (II level)	3.2	

DW = Dry Weight

For each fortification level, the mean recovery and the precision (RSD, relative standard deviation) are in

compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.

The LOQ for acetamiprid in soil was assessed in this study at 0.05 mg/kg (referred to dry soil).

Limit of detection (LOD)

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.

For the analysis of acetamiprid in soil the LOD is 0.0116 mg/kg (referred to dry soil). This value, calculated from the acetamiprid concentration corresponding to the lowest calibration point, is below 30 % of LOQ.

Matrix effects

To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of acetamiprid at 4 ng/mL (nearest to the nominal concentration for the LOQ level) was compared to acetamiprid in solvent at the same concentration. The results are summarized in the following table.

Matrix	Transition	Mean Matrix response Over solvent response %	Matrix effects %
Soil	223/126 (Target)	96	-4
	223/90 (Qualifier)	91	-9

Matrix effects for acetamiprid in soil matrix were found not significant (< 20 %) for both acquired transitions, nevertheless matrix matched calibration standards were used in the quantification of samples for better accuracy.

Confirmation

The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.

The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg _{DW})	Recoveries (%)	Mean Recovery (%)	RSD (%)	Overall mean recovery ± RSD %
0.05 (LOQ)	105.4 106.1 94.1 96.9 100.6	100.6	5.2	97.9 ± 5.2
350 (II level)	92.2 93.4 93.9 95.1 101.1	95.1	3.7	

DW = Dry Weight

Also, for the confirmatory transition, the mean recovery was in the range 70 – 120 % and the precision (RSD, relative standard deviation) ≤ 20 %, in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Stability of final extracts and reference item solutions

During the analytical sequences the injection of intermediate standard solutions and QC samples (recover-

ies) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.

The final extracts were analysed within 24 hours form extraction. The stability of the extracts during the analysis was also proven by the acceptability of recoveries performed concurrently with the samples analysis. Moreover, one recovery at LOQ was verified for storage stability, confirming that the extract is stable (+1.5 %) in refrigerated conditions (< 10 °C) in the dark for 4 days.

The acetamiprid reference item stock solution was proven to be stable for 32 days after preparation at ≤ -18 °C in the dark in study 23128-02R: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ -18 °C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.2.

Conclusion

The method was fully validated according to SANTE/2020/12830 rev.2. The data presented in this report confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of acetamiprid active ingredient in soil samples in the range 0.05 – 350 mg/kg_{DW}.

A 1.1.1.1.11 UHPLC-MS/MS (in artificial soil)

A 1.1.1.1.11.1 Method validation

Comments of zRMS:	Method is accepted
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Reference:	KCP 5.1.2/11 (filled as KCP 10.4.2.1/02)
Report	Collembolan (<i>Folsomia candida</i>) Reproduction Test in soil, Szlauer S., 2024, report no. ETOX-2024-2
Guideline(s):	Yes SANTE/2020/12830, Rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was validated according to SANTE/2020/12830, Rev.2.

The concentration of Acetamiprid in the artificial soil was determined using a ultrahigh performance liquid chromatographic method with tandem mass spectrometry detection (UHPLC-MS/MS). The analytical method involves extracting the sample with water, acetonitrile and a mixture of salts and analysing using UHPLC-MS/MS.

The linearity of response of the analytical method, precision, recovery, limit of quantification (LOQ), detection (LOD), matrix effects and specificity were assessed in the process of the. Sample was analysed within 24 h after collection so sample stability were not assessed in the process of the analytical method validation. The standard solutions were prepared fresh on the day of analysis, so the standard stability was not assessed during the analytical method validation process.

Sample preparation

At least 2 g of sample should be taken for chemical analysis. 4 mL of water and 4 mL of acetonitrile were added to 1.3 g \pm 0.3 g of sample and vigorously shaken by hand (approximately 1 min). Then 2.6 g \pm 0.2

g of the salt mixture (8:2:2:1, w:w:w:w, magnesium sulfate anhydrous : sodium chloride : sodium citrate dihydrate : sodium hydrogen citrate sesquihydrate) was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 rcf). The upper phase was collected. If the concentration of Acetamiprid in the upper phase is greater than the concentration of the highest standard solution used to prepare the calibration curve, this sample should be diluted with acetonitrile so that the estimated concentration is within the range of the calibration curve after this dilution. The collected samples are then analysed using UHPLC-MS/MS.

Chromatographic conditions

UHPLC-MS/MS Agilent Infinity 1290: HiP Sampler G4226A, Binary Pump G4220A, Column Comp. G1316C, Guard Column Zorbax SB-C18 2.1×5 mm, 1.8 µm, Column Zorbax SB-C18 RRHT 2.1×50 mm, 1.8 µm, 600 bar, 6460 Triple Quad Mass Spectrometer (Ion Source AJS ESI)

Injection 2.5 µL

Elution Isocratic

Mobile phases 77%: Water + formic acid (0.05%),
23%: Acetonitrile + formic acid (0.05%)

Flow [mL/min] 0.4

Stop time 2.3 min

Column temperature 40°C

MS/MS	Gas Temperature [°C]	350
	Gas Flow [L/min]	10
	Nebulizer [psi]	45
	Sheath Gas Heater [°C]	350
	Sheath Gas Flow [L/min]	12
	Capillary [V]	2500
	Nozzle Voltage [V]	0

Transition	Precursor m/z	Product m/z	Dwell	Frag [V]	CE [V]	Cell Acc [V]	Polarity
Target	223.1	→ 126	200	100	22	1	Positive
Qualifier	223.1	→ 73.1	200	100	70	1	Positive

Ion ratio [%] 52.2% ± 30% (relative)

Integrator Agile 2 or manual integration if needed.

Validation

Matrix effects

Assessment of matrix effects were performed by comparing the analyte response of the 10 µg/L standard solution of acetamiprid to spiked matrix blank sample at the same concentration.

Preparation of the spiked matrix blank sample: 1.2333 g of dry artificial soil, 4 mL of water and 4 mL of acetonitrile were mixed. Then 2.6167 g of the salt mixture was added, vigorously shaken by hand and centrifuged (5 min, 5000 rcf). 990 µL of the upper phase was collected, 10 µL of the 1 mg/L Acetamiprid standard solution in acetonitrile was added.

Solution	Measurement repetition	Response (area)	Mean Response	Standard deviation	Relative standard deviation [%]
Standard	1	23070	23619	529	2.2
	2	23663			
	3	24126			
Spiked matrix blank	1	24262	24310	95	0.4
	2	24419			
	3	24248			

The matrix effect was calculated as follow:

$$100\% \times \left(\frac{\text{Response (Spiked Matrix Blank Extract)}}{\text{Response (Standard solution)}} - 1 \right) = 100\% \times \left(\frac{24310}{23619} - 1 \right) = 2.9\%$$

The matrix effect did not exceed $\pm 20\%$, so it is not considered significant.

Linearity

Linearity was determined by preparing a series of standard solutions of Acetamiprid at the concentrations of 0.3, 1, 3, 10, 20 and 30 $\mu\text{g/L}$.

Standard solutions were prepared by adding a given volume of Acetamiprid stock solution to a volumetric flask and making up to the mark with acetonitrile.

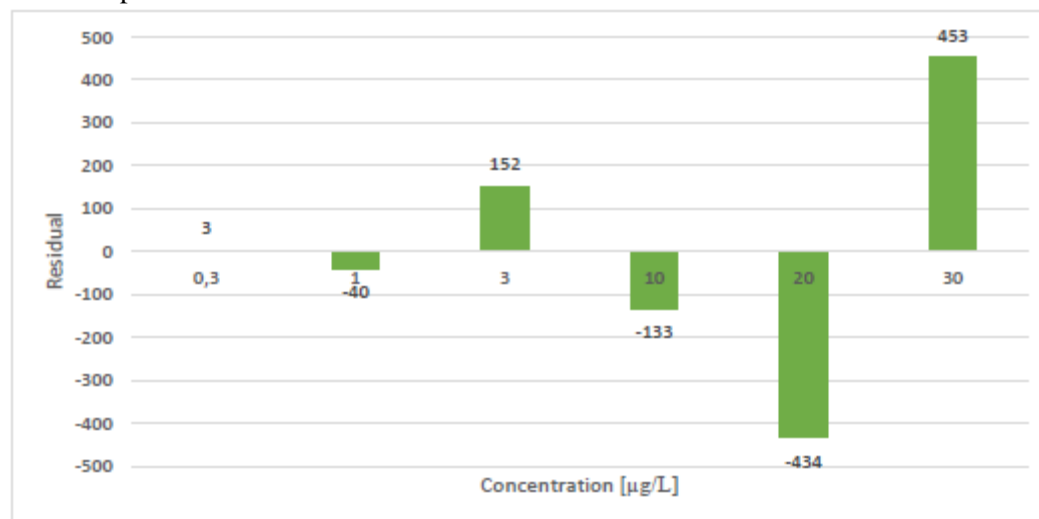
Stock solution 1 g/L was prepared by weighing 10 mg of the Acetamiprid standard and dissolving it in 10 mL of acetonitrile in a 10 mL volumetric flask.

Stock solution 1 mg/L was prepared by taking 10 μL of 1 g/L Acetamiprid stock solution into a 10 mL volumetric flask and making up to the volumetric mark with acetonitrile.

Each solution was analysed 3 times.

Calibration curve equation	Coefficient of determination R^2	Linearity range
$A = 2363 C + 120$	0.9999	0.3 – 30 $\mu\text{g/L}$

Residual plot is shown below:



Precision, accuracy, LOQ and LOD

The Limit of Quantification (LOQ) was determined as the lowest concentration of a detected substance at which the acceptable mean recovery is obtained (70 – 120% with a relative standard deviation (RSD) $\leq 20\%$). The calculated Limit of Detection (LOD) was 30% of the LOQ.

LOQ: 4 µg/L

LOD: 1.2 µg/L

Precision and accuracy were determined at 2 concentration levels of the Test Item Acetamipryd 200 SL in artificial soil: 0.0243 mg/kg (LOQ) and 0.243 mg/kg (10×LOQ).

Five LOQ samples and five 10×LOQ samples were prepared. Sample preparation and analysed 3 times on the same day.

The mean (recovery) and the RSD (repeatability) were calculated from the average recoveries for each concentration level. The outlier was checked using the test Q ($\alpha = 0.95$). No outliers were found. Results are shown in the table below.

Determined precision 6.7% (n = 10) meets the acceptance criteria ($\leq 20\%$).

Determined accuracy 96.0% (n = 10) meets the acceptance criteria (70-120%).

Sample	Concentration Level	Recovery (n=5) [%]	RSD (n=5) [%]	Recovery (n=10) [%]	RSD (n=10) [%]
Test Item	10×LOQ	95.4	5.6	96.0	6.7
	LOQ	96.6	8.2		

Specificity

Representative chromatograms of standard at the lowest calibrated level (LOD), matrix blanks and samples fortified at the LOQ level are provided to prove selectivity of the method.

Preparation of two matrix blank samples: 1.1830 g or 1.4006 g of dry artificial soil was weighted. Then 4 mL of water and 4 mL of acetonitrile were added and vigorously shaken by hand (approximately 1 min). Then 2.5778 g or 2.6011 g of the salt mixture was added, vigorously shaken by hand (approximately 1 min) and centrifuged (5 min, 5000 rcf).

Preparation of the matrix spiked sample at LOQ level (1 µg/L): 10 µL of 100 µg/L Acetamiprid standard solution was added to 0.990 mL of the matrix blank sample.

The 0.3 µg/L concentration corresponds to the lowest concentration on the calibration curve and is equal to 30% of the 1 µg/L concentration. The 1 µg/L concentration is correspond to extract from 1 g dry artificial soil at LOQ level (0.004 mg/kg).

The Y-axis scale on all chromatograms was matched to the chromatogram of the matrix spiked sample at LOQ level.

No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed.

To further confirm the specificity of the analytical method, two ion transitions were recorded:

Target: 223.1 → 126

Qualifier: 223.1 → 73.1

Specificity was verified using the ion transition ratio of $52.2\% \pm 30\%$ (relative). Specificity of the method was confirmed.

Characteristics for the analytical method used for validation of acetamiprid residues in artificial soil

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed. Specificity was verified using the ion transition ratio of $52.2\% \pm 30\%$ (relative). Specificity of the method was confirmed.
Calibration (type, number of data points)	Calibration curve equation: $A = 2363 C + 120$ Coefficient of determination R^2 : 0.9999 Number of data points: 6

Specificity	No signal of the detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. The specificity of the method was confirmed. Specificity was verified using the ion transition ratio of $52.2\% \pm 30\%$ (relative). Specificity of the method was confirmed.
	Linearity range: 0.3 – 30 µg/L
Assessment of matrix effects is presented	yes
Limit of determination/quantification	LOQ: 4 µg/L LOD: 1.2 µg/L

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance acetamiprid in artificial soil.

A 1.1.1.2 Description of analytical methods used in residue studies

A 1.1.1.2.1 LC-MS/MS (in oilseed rape (seed, plant))

A 1.1.1.2.1.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/12

Report VALIDATION STUDY REPORT

Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant), Niewelt-Stasiak S., 2023, Validation Study No: VAL/10/2023

Guideline(s): SANTE/2020/12830 Rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to validate an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl (IM-2-1) in oilseed rape (seed, plant). Specimen extraction and determination of residues was performed using QuEChERS technique.

Quantification was performed by use of LC-MS/MS detection system.

The method was validated over the concentration range of 0.005-0.05 mg/kg (µg/g) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). The limit of detection (LOD) that was expressed as the lowest calibration standard was 0.001 mg/kg for acetamiprid and 0.001 mg/kg for acetamiprid-N-desmethyl (IM-2-1), what corresponds to 0.0005 µg/mL (because weigh of sample was equal 5 g).

This study was conducted to fulfil data requirements outlined in the SANTE/2020/12830 Rev.2. 14. February 2023.

Validation was carried out using untreated raw agricultural commodity material, that was spiked with

active substance at three different concentration levels (LOD, LOQ and 10 x LOQ). Linearity, specificity, precision, recovery, expanded uncertainty and the limit of quantification were determined.

Chromatographic conditions

Instrument settings:

- Liquid Chromatograph LCMS-8050 Shimadzu (WP-132/K/S/ko/LF) consists of:
- Degazer DGU-403
- Two pumps LC-40D XR
- Autosampler SIL-40C XR
- Column oven CTO-40S
- Compressor, generator PEAK Genius 1051
- HPLC Column – Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, Lot no.: B20115

Pumps:

- Mode – Binary gradient
- Total Flow – 0.3 mL/min
- Mobile Phase A – 0.10 % formic acid in water
- Mobile Phase B – 0.10 % formic acid in acetonitrile
- A Conc – 80 %
- B Conc – 20%

Gradient:

Time	Module	Command	Value %
0.01	Pumps	Pump B Conc.	20
10.00	Pumps	Pump B Conc.	90
14.00	Pumps	Pump B Conc.	95
14.01	Pumps	Pump B Conc.	20
16.00	Pumps	Pump B Conc.	20

- Cooler Temp. 4°C
- Oven Temp. 40°C
- Interface: ESI
- Interface heater: on
- Interface Temp.: 300°C
- DL Temperature: 250°C
- Nebulizing Gas Flow: 3.00 L/min
- Heating Gas: On
- Heating Gas Flow: 10.0 L/min
- Heat Block: 400°C
- Drying Gas: On
- Drying Gas Flow: 10.0 L/min

Acetamiprid

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 0.00 min
- End Time: 8.00 min
- Retention Time: 5.33 min

Acetamiprid-N-desmethyl

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 4.75 min

Acetamiprid D3

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 5.32 min

Extraction of acetamiprid in oilseed rape seed

5 g of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of acetonitrile and 10 mL of deionized water was added together with 50 µL of internal standard solution, and the mixture was shaken vigorously by hand for one minute. After addition of buffering salts (4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate, 0.5 g disodium hydrogencitrate sesquihydrate), the mixture was shaken by hand again intensively for 1 min, then centrifuged at 4700 rpm for 5 min for phase separation. Afterwards, 6 mL of the supernatant was transferred to a polypropylene centrifuge tube containing cleanup mixture (900 mg of anhydrous magnesium sulphate, 150 mg of C18, 150 mg of PSA), next the mixture was shaken again intensively for 0.5 min, then centrifuged at 4700 rpm for 5 min for phase separation. After that, the extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis.

Quantification was performed using an internal standard, which was added to the extract after the initial addition of acetonitrile.

Fortification samples

For analytical sequence two samples blank matrix, two samples at limit of detection (LOD), five procedural recoveries at the level of LOQ and five at the level 10 x LOQ were prepared.

5 g of the homogenized untreated sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added, and the sample was extracted as described above.

Fortification level	Amount of standard solution 1.1 added [µL]	Amount of standard solution 1.2 added [µL]	Amount of standard solution 1.3 added [µL]
Matrix blank	-	-	-
PK 0.001 mg/kg (LOD)	-	-	50
PK 0.005 mg/kg (LOQ)	-	25.0	-
PK 0.05 mg/kg (10 x LOQ)	25.0	-	-

Extraction of oilseed rape (seed and plant) samples was performed on 06.06.2023 and after that the samples were directly employed for LC-MS/MS analysis, that was started on the same day.

Duration of the extraction process of oilseed rape (seed, plant) was about 2 h and duration of the chromatographic analysis was 800 min (13.3 h), the total analytical procedure was performed and completed within 1 day.

As required in SANTE/2020/12830 Rev.2. 14. February 2023, if the extracts contain an IL-IS (isotopically labelled internal standard) for quantification, testing of final extract stability is not required since the IL-IS will compensate for losses during extract storage. In case of method without isotopically labelled internal standard if the recoveries in the fortified samples are within the acceptable range of 70-120 % and final extracts are analysed within 24 h, then stability is sufficiently proven.

As there was used IL-IS (for acetamiprid) in this validation study, in addition total analytical procedure was performed and completed within 24h, and recoveries in the fortified samples are within the acceptable range of 70-120 %, stability is sufficiently proven.

Working standards that were used for quantification were always prepared in organic solvent on the same day as the work up of the specimen for residue analysis took place (then standards stability should not be considered to be an issue). However, additional injection of calibration standard was performed in the end of sequence.

Validation

Specificity/selectivity

LC-MS/MS method was used during the study. Two mass transition were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for two matrix blank samples.

Linearity

The linearity of the detector response was demonstrated by single determination of calibration standards at six concentration levels ranging from 0.5 to 500 ppb for acetamiprid and acetamiprid-N-desmethyl. The coefficients of determination (R^2) were determined.

Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than or equal to 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration.

In addition, suitability of the chosen function was demonstrated by a residual analysis using the residuals. The regression residual d_i describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - y_{yi}$$

where:

y_i - the measured value i

y_{yi} - the estimated value which corresponds to y_i and is derived from the calibration function.

Precision, accuracy and uncertainty

Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level.

The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60–120% with $RSD \leq 30\%$, and recovery at fortification level of 0.10 mg/kg (10xLOQ) should be in the range of 70–120% with $RSD \leq 20\%$. RSD were determined only during validation process.

Matrix Effect

In accordance with SANTE/2020/12830 Rev.2. 14. February 2023, assessment of matrix effects should be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix. Matrix effects, expressed in % enhancement or suppression can be evaluated according to the following equation:

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

Matrix effects are considered significant if they exceed $\pm 20\%$.

Matrix	Data Filename	Area	Matrix effect [%]
Acetamiprid in oilseed rape (seed)	OSR seed cal 100 ppb.lcd	5 028 968	-54.8
	cal 100 ppb in solvent.lcd (standard solution in solvent)	11 120 735	
Acetamiprid-N-desmethyl in oilseed rape (seed)	OSR seed cal 100 ppb.lcd	2 347 506	-65.6
	cal 100 ppb in solvent.lcd (standard solution in solvent)	6 818 903	
Acetamiprid in oilseed	OSR plant cal 100 ppb.lcd	8 535 721	-37.6

rape (plant)	cal 100 ppb in solvent.lcd (standard solution in solvent)	13 687 606	
Acetamiprid-N-desmethyl in oilseed rape (plant)	OSR plant cal 100 ppb.lcd	4 093 099	-34.8
	cal 100 ppb in solvent.lcd (standard solution in solvent)	6 279 776	

For acetamiprid and acetamiprid-N-desmethyl matrix in oilseed seed and plant matrix effect calculated using equation exceed $\pm 20\%$. To compensate matrix effect, there was used matrix-matched calibrations.

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

The LOQ is the lowest validated fortification level for which an average recovery in the range of 70-120% (60-120 % in case of level ≤ 0.01 mg/kg) and RSD $\leq 20\%$ ($\leq 30\%$ in case of level ≤ 0.01 mg/kg) is achieved.

LOQ was successfully established at 0.005 mg/kg for acetamiprid and 0.005 mg/kg for acetamiprid-N-desmethyl (IM-2-1), and for ‘sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid’ limit of quantification was 0.01 mg/kg.

The limit of detection (LOD) was estimated at 0.001 mg/kg, for acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant).

Stability of solutions

One of calibration standard (cal 100 ppb) was additionally analysed in the end of sequence to prove stability of standards.

Confirmation

For analyte, two mass transitions were evaluated and used for quantification. A third mass transition was monitored for confirmation of peak identity but was not used for quantification.

Conclusion

The analytical method for determining the residues of acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant) meets the criteria of SANTE/2020/12830 Rev.2. 14. February 2023 documents in terms of precision, accuracy and uncertainty.

The method was validated over the concentration range of 0.005-0.05 mg/kg ($\mu\text{g/g}$) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). Limit of detection was established at 0.001 mg/kg.

Summary of validation results for acetamiprid in oilseed rape

Parameter	Matrix	Criterion of acceptance	Results obtained for acetamiprid (transition 223.10→126.00)	Results obtained for acetamiprid (transition 223.10→56.10)
Specificity/selectivity	seed	fulfilled		
	plant	fulfilled		
Linearity	seed	$R^2 \geq 0.98$	$R^2 = 0.9999$	$R^2 = 0.9999$
	plant		$R^2 = 0.9997$	$R^2 = 0.9998$
Quantification (target) ion	seed	-	223.10→126.00	223.10→56.10
	plant		223.10→126.00	223.10→56.10
Qualification (ref) Ion(s)	seed	-	223.10→56.10 223.10→98.90	-
	plant		223.10→56.10 223.10→98.90	-

Limit of quantification (LOQ)	seed	-	0.005 mg/kg	0.005 mg/kg
	plant		0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	seed	-	0.001 mg/kg	0.001 mg/kg
	plant		0.001 mg/kg	0.001 mg/kg

Summary of validation results for acetamiprid-N-desmethyl (IM-2-1) in oilseed rape

Parameter	Matrix	Criterion of acceptance	Results obtained for acetamiprid-N-desmethyl (transition 210.90→128.10)	Results obtained for acetamiprid-N-desmethyl (transition 208.80→73.10)
Specificity/selectivity	seed	fulfilled		
	plant	fulfilled		
Linearity	seed	$R^2 \geq 0.98$	$R^2 = 0.9998$	$R^2 = 0.9997$
	plant		$R^2 = 0.9981$	$R^2 = 0.9989$
Quantification (target) ion	seed	-	210.90→128.10	208.80→73.10
	plant		210.90→128.10	208.80→73.10
Qualification (ref) Ion(s)	seed	-	208.80→73.10	-
	plant		208.80→73.10	-
Limit of quantification (LOQ)	seed	-	0.005 mg/kg	0.005 mg/kg
	plant		0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	seed	-	0.001 mg/kg	0.001 mg/kg
	plant		0.001 mg/kg	0.001 mg/kg

A 1.1.1.2.2 LC-MS/MS (in potato)

A 1.1.1.2.2.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/13

Report VALIDATION STUDY REPORT
Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in potato, Niewelt-Stasiak S., 2023, Validation Study No: VAL/11/2023

Guideline(s): SANTE/2020/12830 Rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to validate an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in potato. Specimen extraction and determination of residues was performed using QuEChERS technique.

Quantification was performed by use of LC-MS/MS detection system.

The method was validated over the concentration range of 0.005-0.05 mg/kg ($\mu\text{g/g}$) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). The limit of detection (LOD) that was expressed as the lowest calibration standard was 0.001 mg/kg for acetamiprid and 0.001 mg/kg for acetamiprid-N-desmethyl (IM-2-1).

This study was conducted to fulfil data requirements outlined in the SANTE/2020/12830 Rev.2. 14. February 2023.

Validation was carried out using untreated raw agricultural commodity material, that was spiked with active substance at three different concentration levels (LOD, LOQ and 10 x LOQ). Linearity, specificity, precision, recovery, expanded uncertainty and the limit of quantification were determined.

Chromatographic conditions

Instrument settings:

- Liquid Chromatograph LCMS-8050 Shimadzu (WP-132/K/S/ko/LF) consists of:
- Degazer DGU-403
- Two pumps LC-40D XR
- Autosampler SIL-40C XR
- Column oven CTO-40S
- Compressor, generator PEAK Genius 1051
- HPLC Column – Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, Lot no.: B20115

Pumps:

- Mode – Binary gradient
- Total Flow – 0.3 mL/min
- Mobile Phase A – 0.10 % formic acid in water
- Mobile Phase B – 0.10 % formic acid in acetonitrile
- A Conc – 80 %
- B Conc – 20%

Gradient:

Time	Module	Command	Value %
0.01	Pumps	Pump B Conc.	20
10.00	Pumps	Pump B Conc.	90
14.00	Pumps	Pump B Conc.	95
14.01	Pumps	Pump B Conc.	20
16.00	Pumps	Pump B Conc.	20

- Cooler Temp. 4°C
- Oven Temp. 40°C
- Interface: ESI
- Interface heater: on
- Interface Temp.: 300°C
- DL Temperature: 250°C
- Nebulizing Gas Flow: 3.00 L/min
- Heating Gas: On
- Heating Gas Flow: 10.0 L/min
- Heat Block: 400°C
- Drying Gas: On
- Drying Gas Flow: 10.0 L/min

Acetamiprid

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 0.00 min
- End Time: 8.00 min
- Retention Time: 4.98 min

Acetamiprid-N-desmethyl

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 4.41 min

Acetamiprid D3

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 4.962 min

Extraction of acetamiprid in oilseed rape seed

10 g of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of acetonitrile was added together with 100 µL of internal standard solution, and the mixture was shaken vigorously by hand for one minute. After addition of buffering salts (4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dehydrate, 0.5 g disodium hydrogencitrate sesquihydrate), the mixture was shaken by hand again intensively for 1 min, then centrifuged at 4700 rpm for 5 min for phase separation. Afterwards, 6 mL of the supernatant was transferred to a polypropylene centrifuge tube containing of cleanup mixture (900 mg of anhydrous magnesium sulphate, 150 mg of C18, 150 mg of PSA), next the mixture was shaken again by hand intensively for 0.5 min, then centrifuged at 4700 rpm for 5 min for phase separation. After that, the extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis. Quantification was performed using an internal standard, which was added to the extract after the initial addition of acetonitrile.

Fortification samples

For analytical sequence two samples blank matrix, two samples at limit of detection (LOD), five procedural recoveries at the level of LOQ and five at the level 10 x LOQ were prepared.

10 g of the homogenized untreated sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added, and the sample was extracted as described above.

Fortification level	Amount of standard solution 1.1 added [µL]	Amount of standard solution 1.2 added [µL]	Amount of standard solution 1.3 added [µL]
Matrix blank	-	-	-
PK 0.001 mg/kg (LOD)	-	-	100.0
PK 0.005 mg/kg (LOQ)	-	50.0	-
PK 0.05 mg/kg (10 x LOQ)	50.0	-	-

Extraction of all samples was performed on 13.06.2023 and after that the samples were directly employed for LC-MS/MS analysis, that was started on the same day.

Duration of the extraction process was about 2 h and duration of the chromatographic analysis was 400 min (6.7 h), the total analytical procedure was performed and completed within 1 day.

As required in SANTE/2020/12830 Rev.2. 14. February 2023, if the extracts contain an IL-IS (isotopically labelled internal standard) for quantification, testing of final extract stability is not required since the IL-IS will compensate for losses during extract storage. In case of method without isotopically labelled internal standard if the recoveries in the fortified samples are within the acceptable range of 70-120 % and final extracts are analysed within 24 h, then stability is sufficiently proven.

As there was used IL-IS (for acetamiprid) in this validation study, in addition total analytical procedure was performed and completed within 24h, and recoveries in the fortified samples are within the acceptable range of 70-120 %, stability is sufficiently proven.

Working standards that were used for quantification were always prepared in organic solvent on the same day as the work up of the specimen for residue analysis took place (then standards stability should not be considered to be an issue). However, additional injection of calibration standard was performed in the end of sequence.

Validation

Specificity/selectivity

LC-MS/MS method was used during the study. Two mass transitions: 223.10>126.00 and 223.10>56.10 for acetamiprid, 210.90>128.10 and 208.80>73.10 for acetamiprid-N-desmethyl, were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for two matrix blank samples.

Linearity

The linearity of the detector response was demonstrated by single determination of calibration standards at six concentration levels ranging from 1 to 500 ppb for acetamiprid and acetamiprid-N-desmethyl.

The coefficients of determination (R^2) were determined.

Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than or equal to 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration.

In addition, suitability of the chosen function was demonstrated by a residual analysis using the residuals. The regression residual d_i describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - \hat{y}_i$$

where:

y_i - the measured value i

\hat{y}_i - the estimated value which corresponds to y_i and is derived from the calibration function.

Precision, accuracy and uncertainty

Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level.

The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60–120% with $RSD \leq 30\%$, and recovery at fortification level of 0.10 mg/kg (10xLOQ) should be in the range of 70–120% with $RSD \leq 20\%$. RSD were determined only during validation process.

Matrix Effect

In accordance with SANTE/2020/12830 Rev.2. 14. February 2023, assessment of matrix effects should be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix. Matrix effects, expressed in % enhancement or suppression can be evaluated according to the following equation:

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

Matrix effects are considered significant if they exceed $\pm 20\%$.

Matrix	Data Filename	Area	Matrix effect [%]
Acetamiprid	potato cal 100 ppb.lcd	6 698 130	-44.6
	cal 100 ppb in solvent.lcd (standard solution in solvent)	12 097 249	
Acetamiprid-N-desmethyl	potato cal 100 ppb.lcd	4 304 719	-38.1
	cal 100 ppb in solvent.lcd (standard solution in solvent)	6 956 213	

For acetamiprid and acetamiprid-N-desmethyl matrix in oilseed seed and plant matrix effect calculated using equation exceed $\pm 20\%$. To compensate matrix effect, there was used matrix-matched calibrations.

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

The LOQ is the lowest validated fortification level for which an average recovery in the range of 70–120% (60–120% in case of level ≤ 0.01 mg/kg) and RSD $\leq 20\%$ ($\leq 30\%$ in case of level ≤ 0.01 mg/kg) is achieved.

LOQ was successfully established at 0.005 mg/kg for acetamiprid and 0.005 mg/kg for acetamiprid-N-desmethyl (IM-2-1), and for ‘sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid’ limit of quantification was 0.01 mg/kg.

The limit of detection (LOD) was estimated at 0.001 mg/kg, for acetamiprid and acetamiprid-N-desmethyl in potato.

Stability of solutions

One of calibration standard (cal 100 ppb) was additionally analysed in the end of sequence to prove stability of standards.

Confirmation

For analyte, two mass transitions were evaluated and used for quantification. A third mass transition was monitored for confirmation of peak identity but was not used for quantification.

Conclusion

The analytical method for determining the residues of acetamiprid and acetamiprid-N-desmethyl in potato meets the criteria of SANTE/2020/12830 Rev.2. 14. February 2023 (1) documents in terms of precision, accuracy and uncertainty.

The method was validated over the concentration range of 0.005-0.05 mg/kg ($\mu\text{g/g}$) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). Limit of detection was established at 0.001 mg/kg.

Summary of validation results for acetamiprid in potato

Parameter	Criterion of acceptance	Results obtained for acetamiprid (transition 223.10→126.00)	Results obtained for acetamiprid (transition 223.10→56.10)
Specificity/selectivity	fulfilled		
Linearity	$R^2 \geq 0.98$	$R^2 = 0.9996$	$R^2 = 0.9998$
Quantification (target) ion	-	223.10→126.00	223.10→56.10
Qualification (ref) Ion(s)	-	223.10→56.10 223.10→98.90	-

Limit of quantification (LOQ)	-	0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	-	0.001 mg/kg	0.001 mg/kg

Summary of validation results for acetamiprid-N-desmethyl (IM-2-1) in potato

Parameter	Criterion of acceptance	Results obtained for acetamiprid-N-desmethyl (transition 210.90→128.10)	Results obtained for acetamiprid-N-desmethyl (transition 208.80→73.10)
Specificity/selectivity	fulfilled		
Linearity	$R^2 \geq 0.98$	$R^2 = 0.9996$	$R^2 = 0.9997$
Quantification (target) ion	-	210.90→128.10	208.80→73.10
Qualification (ref) Ion(s)	-	208.80→73.10	-
Limit of quantification (LOQ)	-	0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	-	0.001 mg/kg	0.001 mg/kg

A 1.1.1.2.3 LC-MS/MS (in apple)

A 1.1.1.2.3.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/14

Report VALIDATION STUDY REPORT
Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in apple, Niewelt-Stasiak S., 2023, Validation Study No: VAL/12/2023

Guideline(s): SANTE/2020/12830 Rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objective of the study was to validate an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in apple. Specimen extraction and determination of residues was performed using QuEChERS technique.

Quantification was performed by use of LC-MS/MS detection system.

The method was validated over the concentration range of 0.005-0.05 mg/kg (µg/g) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). The limit of detection (LOD) that was expressed as the lowest calibration standard was 0.001 mg/kg for acetamiprid and 0.001 mg/kg for acetamiprid-N-desmethyl (IM-2-1).

This study was conducted to fulfil data requirements outlined in the SANTE/2020/12830 Rev.2. 14. February 2023.

Validation was carried out using untreated raw agricultural commodity material, that was spiked with active substance at three different concentration levels (LOD, LOQ and 10 x LOQ). Linearity, specificity,

precision, recovery, expanded uncertainty and the limit of quantification were determined.

Chromatographic conditions

Instrument settings:

- Liquid Chromatograph LCMS-8050 Shimadzu (WP-132/K/S/ko/LF) consists of:
- Degazer DGU-403
- Two pumps LC-40D XR
- Autosampler SIL-40C XR
- Column oven CTO-40S
- Compressor, generator PEAK Genius 1051
- HPLC Column – Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, Lot no.: B20115

Pumps:

- Mode – Binary gradient
- Total Flow – 0.3 mL/min
- Mobile Phase A – 0.10 % formic acid in water
- Mobile Phase B – 0.10 % formic acid in acetonitrile
- A Conc – 80 %
- B Conc – 20%

Gradient:

Time	Module	Command	Value %
0.01	Pumps	Pump B Conc.	20
10.00	Pumps	Pump B Conc.	90
14.00	Pumps	Pump B Conc.	95
14.01	Pumps	Pump B Conc.	20
16.00	Pumps	Pump B Conc.	20

- Cooler Temp. 4°C
- Oven Temp. 40°C
- Interface: ESI
- Interface heater: on
- Interface Temp.: 300°C
- DL Temperature: 250°C
- Nebulizing Gas Flow: 3.00 L/min
- Heating Gas: On
- Heating Gas Flow: 10.0 L/min
- Heat Block: 400°C
- Drying Gas: On
- Drying Gas Flow: 10.0 L/min

Acetamiprid

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 0.00 min
- End Time: 8.00 min
- Retention Time: 4.98 min

Acetamiprid-N-desmethyl

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 4.41 min

Acetamiprid D3

- Acquisition Mode: MRM
- Polarity: Positive
- Start time: 1.50 min
- End Time: 8.00 min
- Retention Time: 4.96 min

Extraction of acetamiprid in oilseed rape seed

10 g of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of acetonitrile was added together with 100 µL of internal standard solution, and the mixture was shaken vigorously by hand for one minute. After addition of buffering salts (4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dehydrate, 0.5 g disodium hydrogencitrate sesquihydrate), the mixture was shaken by hand again intensively for 1 min, then centrifuged at 4700 rpm for 5 min for phase separation. Afterwards, 6 mL of the supernatant was transferred to a polypropylene centrifuge tube containing of cleanup mixture (900 mg of anhydrous magnesium sulphate, 150 mg of C18, 150 mg of PSA), next the mixture was shaken again by hand intensively for 0.5 min, then centrifuged at 4700 rpm for 5 min for phase separation. After that, the extract was filtered through a membrane filter and the final extract was directly employed for LC-MS/MS analysis. Quantification was performed using an internal standard, which was added to the extract after the initial addition of acetonitrile.

Fortification samples

For analytical sequence two samples blank matrix, two samples at limit of detection (LOD), five procedural recoveries at the level of LOQ and five at the level 10 x LOQ were prepared.

10 g of the homogenized untreated sample was weighed into a 50 mL centrifuge tube. Appropriate active substance standard solution was added, and the sample was extracted as described above.

Fortification level	Amount of standard solution 1.1 added [µL]	Amount of standard solution 1.2 added [µL]	Amount of standard solution 1.3 added [µL]
Matrix blank	-	-	-
PK 0.001 mg/kg (LOD)	-	-	100.0
PK 0.005 mg/kg (LOQ)	-	50.0	-
PK 0.05 mg/kg (10 x LOQ)	50.0	-	-

Extraction of all samples was performed on 05.06.2023 and after that the samples were directly employed for LC-MS/MS analysis, that was started on the same day.

Duration of the extraction process was about 2 h and duration of the chromatographic analysis was 416 min (4.9 h), the total analytical procedure was performed and completed within 1 day.

As required in SANTE/2020/12830 Rev.2. 14. February 2023, if the extracts contain an IL-IS (isotopically labelled internal standard) for quantification, testing of final extract stability is not required since the IL-IS will compensate for losses during extract storage. In case of method without isotopically labelled internal standard if the recoveries in the fortified samples are within the acceptable range of 70-120 % and final extracts are analysed within 24 h, then stability is sufficiently proven.

As there was used IL-IS (for acetamiprid) in this validation study, in addition total analytical procedure was performed and completed within 24h, and recoveries in the fortified samples are within the acceptable range of 70-120 %, stability is sufficiently proven.

Working standards that were used for quantification were always prepared in organic solvent on the same day as the work up of the specimen for residue analysis took place (then standards stability should not be considered to be an issue). However, additional injection of calibration standard was performed in the end of sequence.

Validation

Specificity/selectivity

LC-MS/MS method was used during the study. Two mass transitions: 223.10>126.00 and 223.10>56.10 for acetamiprid, 210.90>128.10 and 208.80>73.10 for acetamiprid-N-desmethyl, were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for two matrix blank samples.

Linearity

The linearity of the detector response was demonstrated by single determination of calibration standards at six concentration levels ranging from 1 to 500 ppb for acetamiprid and acetamiprid-N-desmethyl.

The coefficients of determination (R^2) were determined.

Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than or equal to 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration.

In addition, suitability of the chosen function was demonstrated by a residual analysis using the residuals. The regression residual d_i describes the vertical distance of measured values from the regression curve according to:

$$d_i = y_i - \hat{y}_i$$

where:

y_i - the measured value i

\hat{y}_i - the estimated value which corresponds to y_i and is derived from the calibration function.

Precision, accuracy and uncertainty

Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level.

The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60–120% with $RSD \leq 30\%$, and recovery at fortification level of 0.10 mg/kg (10xLOQ) should be in the range of 70–120% with $RSD \leq 20\%$. RSD were determined only during validation process.

Matrix Effect

In accordance with SANTE/2020/12830 Rev.2. 14. February 2023, assessment of matrix effects should be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix. Matrix effects, expressed in % enhancement or suppression can be evaluated according to the following equation:

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

Matrix effects are considered significant if they exceed $\pm 20\%$.

Matrix	Data Filename	Area	Matrix effect [%]
Acetamiprid	apple cal 100 ppb.lcd	11 539 189	8.0
	cal 100 ppb in solvent.lcd (standard solution in solvent)	10 683 071	
Acetamiprid-N-desmethyl	apple cal 100 ppb.lcd	2 514 224	-1.9
	cal 100 ppb in solvent.lcd (standard solution in solvent)	2 563 389	

For acetamiprid and acetamiprid-N-desmethyl matrix effects calculated using equation are <20%. Nevertheless, there was used matrix-matched calibration.

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

The LOQ is the lowest validated fortification level for which an average recovery in the range of 70-120% (60-120 % in case of level ≤ 0.01 mg/kg) and RSD $\leq 20\%$ ($\leq 30\%$ in case of level ≤ 0.01 mg/kg) is achieved.

LOQ was successfully established at 0.005 mg/kg for acetamiprid and 0.005 mg/kg for acetamiprid-N-desmethyl (IM-2-1), and for "sum of acetamiprid and N-desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid" limit of quantification was 0.01 mg/kg.

The limit of detection (LOD) was estimated at 0.001 mg/kg, for acetamiprid and acetamiprid-N-desmethyl in apple.

Stability of solutions

One of calibration standard (cal 100 ppb) was additionally analysed in the end of sequence to prove stability of standards.

Confirmation

For analyte, two mass transitions were evaluated and used for quantification. A third mass transition was monitored for confirmation of peak identity but was not used for quantification.

Conclusion

The analytical method for determining the residues of acetamiprid and acetamiprid-N-desmethyl in apple meets the criteria of SANTE/2020/12830 Rev.2. 14. February 2023 (1) documents in terms of precision, accuracy and uncertainty.

The method was validated over the concentration range of 0.005-0.05 mg/kg ($\mu\text{g/g}$) for acetamiprid and 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). Limit of detection was established at 0.001 mg/kg.

Summary of validation results for acetamiprid in apple

Parameter	Criterion of acceptance	Results obtained for acetamiprid (transition 223.10→126.00)	Results obtained for acetamiprid (transition 223.10→56.10)
Specificity/selectivity	fulfilled		
Linearity	$R^2 \geq 0.98$	$R^2 = 0.9998$	$R^2 = 0.9996$
Quantification (target) ion	-	223.10→126.00	223.10→56.10
Qualification (ref) Ion(s)	-	223.10→56.10 223.10→98.90	-
Limit of quantification (LOQ)	-	0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	-	0.001 mg/kg	0.001 mg/kg

Summary of validation results for acetamiprid-N-desmethyl (IM-2-1) in apple

Parameter	Criterion of acceptance	Results obtained for acetamiprid-N-desmethyl (transition 210.90→128.10)	Results obtained for acetamiprid-N-desmethyl (transition 208.80→73.10)
Specificity/selectivity	fulfilled		
Linearity	$R^2 \geq 0.98$	$R^2 = 0.9994$	$R^2 = 0.9995$
Quantification (target) ion	-	210.90→128.10	208.80→73.10

Qualification (ref) Ion(s)	-	208.80→73.10	-
Limit of quantification (LOQ)	-	0.005 mg/kg	0.005 mg/kg
Limit of detection (LOD)	-	0.001 mg/kg	0.001 mg/kg

A 1.1.1.2.4 LC-MS/MS (in honey)

A 1.1.1.2.4.1 Method validation

Comments of Evaluator:	Method is accepted
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Reference: KCP 5.1.2/15 and KCP 5.2/01

Report Determination of Acetamiprid Residues in Honey Following Application on Winter Oilseed Rape with Piorun 200 SL under semi field Conditions in Northern and Southern Europe in 2023, Lefebvre C., 2023 Report No. R C2051

Guideline(s): SANTE/2020/12830, Rev.2

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

The objectives of the analytical phase were:

- Validate the analysis of acetamiprid-N-desmethyl (IM-2-1) in honey.
- Determine residue levels of acetamiprid and its metabolite acetamiprid-N-desmethyl (IM-2-1) expressed as acetamiprid in honey specimens generated during the field phase.

Samples were analysed within 30 days after sampling.

Water was added to the sample (5 g) which was then extracted with acetonitrile/acetic acid 99.9:0.1%. After addition of MgSO₄, NaCl, and buffering citrate salts (pH 5-5.5), the mixture was shaken intensively and centrifuged for phase separation. An aliquot of the organic phase was cleaned-up by:

- dispersive PSA addition,
- MgSO₄ addition.

Before analysis, the extract was diluted with the acetonitrile/acetic acid 99.9:0.1% mixture.

The analysis was done with LC-MS/MS.

The evaluated validation parameters were linearity, recovery and precision, limit of quantification and detection, selectivity, confirmation, matrix effect and stability. This study was conducted to fulfil data requirements outlined in the SANTE/2020/12830 Rev.2. 14. February 2023.

Chromatographic conditions - LC-MS/MS - 6500

Column

Description	BEH C18	Supplier	WATERS	Particles	1.7 µm
Internal diam. x length	2.1 x 50 mm	Supplier reference	186002350	Comment	-
Development Column ANADIAG Number	338	Stationary Phase	C18		

Mobile phase

A =	Water + 0.1% formic acid
B =	Acetonitrile + 0.1% formic acid

Sample temperature	15°C
Column temperature	40°C

Elution

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

*0=linear

Detector

IONISATION mode	<input checked="" type="checkbox"/> ES	<input type="checkbox"/> APCI		
Polarity	<input checked="" type="checkbox"/> Pos	<input type="checkbox"/> Neg		
Collision gas setting (CAD)	Nitrogen set at 7 (arbitrary units)		Gas flow 1 (GS1)	Air set at 40 (arbitrary units)
Curtain gas (CUR)	Nitrogen set at 25 (arbitrary units)		Gas flow 2 (GS2)	Air set at 70 (arbitrary units)
Ionspray turbo heater (TEM)	650 °C		Nebulizer current (µA) (APCI)	-
Capillary voltage (IS)	4500 V		-	-

Active ingredient(s)	Dwell time (ms)	Declustering Potential DP (V)	Entrance Potential EP (V)	Collision Energy CE (V)	Collision Cell Exit Potential CXP (V)	TRANSITIONS
Acetamiprid-N-desmethyl (IM-2-1)	100	31	10	25	14	209.1 > 125.9 **
	100	31	10	43	10	209.1 > 90.0
Acetamiprid	100	36	10	29	16	223.1 > 125.9 **
	100	36	10	47	12	223.1 > 89.9

** quantitation transition

Valco valve

Prog.	Time (min.)	Position	Prog.	Time (min.)	Position
Pg1	0.0	A (Waste)	Pg3	3.9	A (Waste)
Pg2	0.1	B (Detector)	Pg4	-	-

Date of application of analytical conditions: 22/05/2023

Study	C2051	Column ANADIAG number	338
Matrix	Honey	Retention time	Acetamiprid: ≈ 1.6 min. IM-2-1: ≈ 1.5 min
Sample temperature	+15 °C	Injected volume	5 µL

Preparation of reagents solutions and mixtures

Extraction solution: acetonitrile / acetic acid 99.9:0.1%

Transfer 400 mL of HPLC acetonitrile into a 500 mL volumetric flask.

Add 0.5 mL of acetic acid.

Make up to the mark with acetonitrile.

Mix well.

Use within 7 days after preparation.

Mobile phase A: H₂O + 0.1% formic acid

In a 1 L glass bottle, pour 1L of H₂O. Add 1 mL of formic acid.

Mix well.

Use within 7 days after preparation.

Mobile phase B: HPLC acetonitrile + 0.1% formic acid

In a 1 L glass bottle, pour 1L of HPLC acetonitrile. Add 1 mL of formic acid.

Mix well.

Use within 7 days after preparation.

Preparation of standard solutions

Stock solutions (separate analyte)

- Stock solution acetamiprid for fortification (SMF)

Prepare an acetamiprid stock solution at approximately 1 mg/mL by accurately weighing 10 mg of analytical standard into a 10 mL volumetric flask and bringing to volume with acetonitrile.

- Stock solution acetamiprid for calibration (SMC)

Prepare an acetamiprid stock solution at approximately 1 mg/mL by accurately weighing 10 mg of analytical standard into a 10 mL volumetric flask and bringing to volume with acetonitrile.

- Stock solution acetamiprid-N-desmethyl

Use the commercial solution at 100 µg/mL in acetonitrile as stock solution.

Spiking solutions (analytes together)

Prepare fortification solution containing acetamiprid and acetamiprid-N-desmethyl at 5 and 0.5 µg/mL by dilution of the acetamiprid stock solution SMF and the acetamiprid-N-desmethyl commercial solution with the extraction solution.

Fortifications were performed by adding known amounts of these spiking solutions to control samples just prior to the extraction step (meaning that working solution of standard is added to the homogenized subsample, before mixing with the extraction solvent).

Calibration solutions

- Intermediate calibration solutions (both analytes together)

Prepare an acetamiprid intermediate calibration solution at 100 µg/mL by dilution of the acetamiprid stock solution SMC with the extraction solution.

Prepare an acetamiprid and acetamiprid-N-desmethyl intermediate calibration solution at 2.5 µg/mL by dilution of the intermediate acetamiprid solution at 100 µg/mL and of the acetamiprid-N-desmethyl stock solution with the extraction solution.

- Matrix-matched Intermediate calibration solutions (both analytes together)

Prepare an acetamiprid and acetamiprid-N-desmethyl intermediate calibration solution at 0.25 µg/mL by dilution of the acetamiprid and acetamiprid-N-desmethyl intermediate solution at 2.5 µg/mL with the control extract.

Prepare an acetamiprid and acetamiprid-N-desmethyl intermediate calibration solution at 0.025 µg/mL by dilution of the acetamiprid and acetamiprid-N-desmethyl intermediate solution at 0.25 µg/mL with the control extract.

- Matrix-matched calibration solutions (both analytes together)

Prepare matrix-matched calibration solutions in the final control extract ranging from ≈0.38 to 15 ng/mL by dilution of matrix-matched intermediates calibration solutions or by dilution of the matrix-matched calibration solution.

- Matrix-effect on calibration solutions testing

Prepare a calibration solution at 12.5 ng/mL by dilution of the intermediate calibration solution of acetamiprid and acetamiprid-N-desmethyl at 2500 ng/mL with the extraction solution

Extraction of samples

Weigh 5.0 (±0.03) g of the sample into a 50 mL centrifuge tube.

Fortify if relevant.

Add 10 mL of ultra-pure water and 10 mL of extraction solution (take into account the volume used to fortify samples if it is greater than 0.5 mL).

and shake manually vigorously for 1 min.
Add the content of a Supel™ QuE citrate Extraction Tube.
Attach the centrifuge tubes on the mechanical shaker for centrifuge tubes.
Shake vigorously for 10 min and centrifuge for 5 min at 3000 rpm.
Adapt the reciprocal speed in order that the bottom of the tube is empty after each rotation.
Transfer about 6 mL of the acetonitrile layer into a 15 mL centrifuge tube.
Transfer into a Supel™ QuE PSA tube.
Attach the centrifuge tubes on the mechanical shaker for centrifuge tubes.
Shake vigorously for 10 min and centrifuge for 5 min at 3000 rpm.
Transfer 0.75 mL aliquot of the supernatant into an Eppendorf tube, add 0.75 mL of extraction solution and mix.
Centrifuge at 13000 rpm for 1 min.
Fill forty autosampler vials; one with about 1 mL and one with about 0.5 mL.
Store the samples frozen before analysis.
Analyse by LC/MS/MS.

Validation

Linearity

The analytical calibration consisted of matrix-matched calibration solutions of acetamiprid and acetamiprid-N-desmethyl (IM-2-1) at least at 5 different levels of concentration, ranged from 0.38 to 15.1 ng/mL (corresponding to 0.0015 to 0.0604 mg/kg).

The calibration covered two orders of magnitude and ranged from $\leq 30\%$ of the LOQ to $\geq 20\%$ above the highest level. Standard concentrations were distributed evenly over the full calibration range.

Calibration curves were run for each analysis sequence for both primary and confirmatory methods.

The linear correlation coefficients were > 0.990 , showing good linearity with regression residuals randomly distributed for both primary and confirmatory methods.

The linearity equation and R^2 correlation factor are reported below.

Matrix	Analyte	Detection	Calibration function:	R^2 correlation
Honey	Acetamiprid	Primary method 223.1 > 125.9	$C (\text{Concentration}) = 1.0379E-05 \times S (\text{Peak area}) - 0.080$	0.99983
		Confirmatory method 223.1 > 89.9	$C (\text{Concentration}) = 5.3925E-05 \times S (\text{Peak area}) - 0.048$	0.99969
	Acetamiprid-N-desmethyl	Primary method 209.1 > 125.9	$C (\text{Concentration}) = 8.5732E-06 \times S (\text{Peak area}) - 0.004$	0.99959
		Confirmatory method 209.1 > 90.0	$C (\text{Concentration}) = 5.7350E-05 \times S (\text{Peak area}) + 0.046$	0.99956

Recovery and repeatability

Primary method - Untreated samples

Two untreated samples were extracted concurrently with fortified samples. Residue levels were reported above the limit of detection. No interferences above 30% of the limit of quantification were recorded.

Primary method - Fortified samples

For the primary method, recovery and repeatability (as precision, % RSD) tests were performed by untreated control samples spiked with acetamiprid and acetamiprid-N-desmethyl before extraction at the following fortification levels:

- LOQ (5 samples),
- 10 x LOQ (5 samples).

For the primary method, recovery and repeatability (as precision, % RSD) data complied with the requirements of the SANTE/2020/12830, Rev.2 guideline as mean recoveries were within the range 60-120% with RSD less than 30% for spiked samples at 0.005 mg/kg and within the range 70-120% with RSD less than 20% for spiked samples at 0.050 mg/kg.

Confirmatory method - Untreated samples

Two untreated samples were extracted concurrently with fortified samples. Residue levels were reported above the limit of detection. No interferences above 30% of the limit of quantification were recorded.

Confirmatory method - Fortified samples

For the confirmatory method, recovery and repeatability (as precision, % RSD) tests were performed by untreated control samples spiked with acetamiprid and acetamiprid-N-desmethyl at the LOQ (5 samples) before extraction.

For the confirmatory method, recovery and repeatability (as precision, % RSD) data complied with the requirements of the SANTE/2020/12830, Rev.2 guideline as mean recoveries were within the range 60-120% with RSD less than 30% for spiked samples at the LOQ level.

Selectivity and specificity

Representative, clearly labelled chromatograms of standard at the lowest calibrated level, untreated sample and sample fortified at the lowest fortification level for each analyte for both primary and confirmatory methods were provided to prove selectivity of the method.

Mass spectra were provided to justify the selection of ions used for determination.

Untreated samples (non-fortified samples) were determined from the matrix used in fortification experiments and were not higher than 30% of the LOQ for both primary and confirmatory methods.

Matrix Effect

Assessment of matrix effects was performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix for both primary and confirmatory methods.

Matrix effects, expressed in % enhancement or suppression were evaluated according to the following equation:

$$\text{Recovery for matrix effect (\%)} = 100 \times \frac{S_m (\text{matrix})}{S (\text{solvent})} - 100$$

$S_m (\text{matrix})$ = average of peak area matrix or peak area (matrix)

$S (\text{solvent})$ = peak area (solvent)

Matrix effects were considered significant if they exceed $\pm 20\%$.

Primary method			
Matrix	Analyte	Concentration (ng/mL)	Matrix effect (%)
Honey	Acetamiprid	12.6	-5.8
	Acetamiprid-N-desmethyl	12.6	-6.3
Confirmatory method			
Matrix	Analyte	Concentration (ng/mL)	Matrix effect (%)
Honey	Acetamiprid	12.6	-5.5
	Acetamiprid-N-desmethyl	12.6	-14.4

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

LOD

The limit of detection is the lowest measurable standard concentration estimated at 3 times the background noise under the analytical conditions used.

The LOD was 0.38 ng/mL for each analyte in honey (corresponding to 0.0015 mg/kg).

LOQ

The limit of quantification has been validated by fortifications at this level.
The LOQ was 0.005 mg/kg for each analyte in honey.

Confirmation

The confirmatory method was required to confirm that the primary method detected the correct analyte (analyte identity) and that the analyte signal of the primary method was quantitatively correct and not affected by any other compound.

Confirmation simultaneously to primary detection:

The confirmatory method was achieved by monitoring 1 additional transition.

	Primary transition	Confirmatory transition
Acetamiprid	m/z 223.1 > 125.9	m/z 223.1 > 89.9
Acetamiprid-N-desmethyl	m/z 209.1 > 125.9	m/z 209.1 > 90.0

Storage stability of extracts and standards solutions

The storage stability of the analytes in final extracts was evaluated by analysing spiked samples after frozen storage.

The storage stability of standard solutions was evaluated by comparing response factors obtained for stored solutions to freshly prepared solutions.

Storage stability of extracts

Spiked samples at 10xLOQ level were stored frozen after samples extraction and analysed against freshly prepared standards to check the stability of the final extracts.

The stability of the analytes in the final extracts was sufficiently proven according to the SAN-TE/2020/12830, Rev.2 guideline, as mean recoveries in the fortified samples were within the range 70-120%, measured against freshly prepared standards.

Acetamiprid and acetamiprid-N-desmethyl residues were stable in honey extracts for at least 9 days of frozen storage.

Storage stability of standards solutions

A matrix-matched standard solution at about 12 ng/mL for acetamiprid and acetamiprid-N-desmethyl was analysed after frozen storage and the average response factor (5 injections) obtained was compared with the average response factor obtained for a freshly prepared solution.

The difference between average response factors from at least 5 replicate measurements for each of the two solutions did not differ by more than 10%.

Acetamiprid and acetamiprid-N-desmethyl residues were stable in honey matrix-matched calibration solutions for at least 9 days of frozen storage.

Conclusion

The methods were fully validated according to SANTE/2020/12830, Rev.2. Results of the validation of analytical method confirm that this method is suitable for analysis residue levels of acetamiprid and its metabolite acetamiprid-N-desmethyl (IM-2-1) expressed as acetamiprid in honey specimens generated during the field phase.

A 1.1.1.2.4.2 Independent laboratory validation

Comments of zRMS:	Method is accepted
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Reference: KCP 5.2/02

Report INDEPENDENT LABORATORY VALIDATION - STUDY REPORT
/draft/
Validation of an analytical method for the determination of residues of acet-

	amiprid and acetamiprid-N-desmethyl in honey, Niewelt-Stasiak S., 2024, Report No. ILV/02/2023
Guideline(s):	Yes Regulation (EC) No 1107/2009 SANTE/2020/12830 Rev.2, 14 February 2023
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of the study was to reproduce independent validation method that was developed by other (primary) laboratory – method for determination of residues of acetamiprid and its metabolite acetamiprid-N-desmethyl (IM-2-1) in honey. Specimen extraction and determination of residues was performed using QuEChERS technique.

Quantification was performed by use of LC-MS/MS detection system. The limit of detection (LOD) that was expressed as the lowest calibration standard was 0.0015 mg/kg for acetamiprid and acetamiprid-N-desmethyl in honey, what corresponds to 0.00038 µg/mL. Limit of quantification (LOQ) of the analytical method was 0.005 mg/kg for acetamiprid and acetamiprid-N-desmethyl in honey.

Validation was carried out using untreated raw agricultural commodity material, that was spiked with active substance at three different concentration levels (LOD, LOQ and 10 x LOQ). Linearity, specificity, precision, recovery, expanded uncertainty and the limit of quantification were determined.

The extracts were analysed using liquid chromatography coupled with mass spectrometry, by single extraction and single injection to the detection system. Final extracts were employed for LC-MS/MS analysis directly after completion of the extraction procedure (on the same day). Data acquisition was carried out in the MRM mode. The analysis was performed using external standard method.

For analytes, two mass transitions were evaluated and used for quantification.

- Acetamiprid (ESI (+)) 223.10 → 125.90 (+)
223.10 → 89.90 (+)
- Acetamiprid-N-desmethyl (ESI (+)) 209.10 → 125.90 (+)
209.10 → 90.00 (+)

LC-MS/MS settings

Instrument settings:

Degazer DGU-403
Two pumps LC-40D XR
Autosampler SIL-40C XR
Column oven CTO-40S
Compressor, generator PEAK Genius 1051
HPLC Column – ACQUITY UPLC BEH C18, 2.1 X 50 mm, 1.7µm; Lot: 0478341431

Pumps:

Mode – Binary gradient
Total Flow – 0.4 mL/min
Mobile Phase A – 0.10 % formic acid in water
Mobile Phase B – 0.10 % formic acid in acetonitrile
A Conc – 90 %
B Conc – 10%

Time	Module	Command	Value %
0.00	Pumps	Pump B Conc.	10

Time	Module	Command	Value %
2.00	Pumps	Pump B Conc.	100
3.20	Pumps	Pump B Conc.	100
3.25	Pumps	Pump B Conc.	10
4.00	Pumps	Pump B Conc.	10

Stop time: 4 min

Cooler Temp.: 15°C

Oven Temp.: 40°C

Curtain Gas: 25.0

Collision Gas: Medium

IonSpray Voltage: 4500.0

Temperature: 650.0

Ion Source Gas 1 (GS1): 40.0

Ion Source Gas 2 (GS2): 70.0

Acetamiprid

Acquisition Mode: MRM

Polarity: Positive

Retention Time: 1.48 min

Acetamiprid-N-desmethyl

Acquisition Mode: MRM

Polarity: Positive

Retention Time: 1.40 min

Results and discussions

Specificity and selectivity

LC-MS/MS method was used during the study. Two mass transition were evaluated and used for quantification. The specificity of the method was evaluated on the basis of the analysis of chromatograms recorded for two matrix blank samples.

Linearity

The linearity of the detector response was demonstrated by single determination of calibration standards at seven concentration levels ranging from 0.38 to 15.1 ppb for acetamiprid and acetamiprid-N-desmethyl in honey.

The coefficients of determination (R^2) were determined.

Linear regression analysis with 1/x weighting was used to describe the detector response as a function of the calibration standard concentrations. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the coefficients of determination (r) were greater than or equal to 0.990 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration

Limit of quantification

The LOQ is the lowest validated fortification level for which an average recovery in the range of 70 – 120% (60 – 120 % in case of level ≤ 0.01 mg/kg) and RSD ≤ 20 % (≤ 30 % in case of level ≤ 0.01 mg/kg) is achieved.

The limit of detection (LOD) was estimated at 0.0015 mg/kg, for acetamiprid and acetamiprid-N-desmethyl in honey.

LOQ was successfully established at 0.005 mg/kg for acetamiprid and acetamiprid-N-desmethyl in honey.

Precision, accuracy and uncertainty

Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five

samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level.

The mean recovery at fortification level of 0.005 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30\%$, and recovery at fortification level of 0.10 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20\%$. RSD were determined only during validation process.

Matrix effects

In accordance to SANTE/2020/12830 Rev. 2, 14 February 2023, assessment of matrix effects should be performed by comparing the analyte response of at least one individual standard prepared in solvent to at least one prepared in blank matrix. Matrix effects, expressed in % enhancement or suppression can be evaluated according to the following equation:

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

Matrix effects are considered significant if they exceed $\pm 20\%$.

In case of this validation study, calibration standard at 12.5 ng/mL in solvent was additionally analysed and matrix effects were calculated according to the equation above. The results are below.

Matrix	Analyte	Matrix effect [%]
Honey	Acetamiprid	-9.8
	Acetamiprid-N-desmethyl (IM-2-1)	-9.7

For acetamiprid and acetamiprid-N-desmethyl in honey matrix effects calculated using equation are $< \pm 20\%$. However, to compensate matrix effects, the matrix-matched calibrations were used.

Stability of solutions

One of calibration standard was additionally analysed in the end of sequence to prove stability of standards.

Table A 1: Recovery results from independent laboratory validation of acetamiprid using the analytical method

Transition: 223.10→125.90

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0046	92.0	0.05	0.048	96.7
	0.0048	95.8		0.048	96.2
	0.0046	92.5		0.048	96.5
	0.0047	94.5		0.048	96.9
	0.0049	97.4		0.047	93.8
Average	0.0047	94.4	Average	0.048	96.0
SD	0.00011	2.25	SD	0.00064	1.29
RSD [%]	2.38		RSD [%]	1.34	
Uncertainty [%]	12.1		Uncertainty [%]	8.4	

Table A 2: Recovery results from independent laboratory validation of acetamiprid using the analytical method

Transition: 223.10 → 89.90

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0047	93.2	0.05	0.048	96.0
	0.0048	96.3		0.048	96.8
	0.0046	92.8		0.048	96.0
	0.0048	96.2		0.048	96.3
	0.0049	98.0		0.047	93.1
Average	0.0048	95.3	Average	0.048	95.6
SD	0.00011	2.22	SD	0.00072	1.43
RSD [%]	2.33		RSD [%]	1.50	
Uncertainty [%]	10.5		Uncertainty [%]	9.2	

Table A 3: Recovery results from independent laboratory validation of acetamiprid-N-desmethyl (IM-2-1) using the analytical method

Transition: 209.10 → 125.90

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0046	91.8	0.05	0.048	95.0
	0.0052	104.6		0.047	94.0
	0.0045	89.4		0.047	93.1
	0.0047	93.9		0.047	94.3
	0.0045	89.8		0.047	93.4
Average	0.0047	93.9	Average	0.047	94.0
SD	0.00031	6.25	SD	0.00037	0.74
RSD [%]	6.66		RSD [%]	0.79	
Uncertainty [%]	18.0		Uncertainty [%]	12.1	

Table A 4: Recovery results from independent laboratory validation of acetamiprid-N-desmethyl (IM-2-1) using the analytical method

Transition: 209.10→90.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0046	91.1	0.05	0.047	94.4
	0.0052	104.8		0.047	94.0
	0.0045	90.4		0.047	94.0
	0.0047	94.6		0.047	94.6
	0.0045	90.9		0.047	93.6
Average	0.0047	94.4	Average	0.047	94.1
SD	0.00030	6.07	SD	0.00020	0.39
RSD [%]	6.43		RSD [%]	0.42	
Uncertainty [%]	17.1		Uncertainty [%]	11.8	

Table A 5: Characteristics for the analytical method used for independent laboratory validation of acetamiprid and its metabolite acetamiprid-N-desmethyl (IM-2-1) residues in honey

	Acetamiprid		Acetamiprid-N-desmethyl (IM-2-1)	
Specificity	fulfilled		fulfilled	
Linearity	<u>transition</u> 223.10→125.90 $R^2 = 0.9999$ $y = 5.04304e5x + 15107.17784$ number of data points = 7	<u>transition</u> 223.10→89.90 $R^2 = 0.9998$ $y = 1.17709e5x + 2410.26673$ number of data points = 7	<u>transition</u> 209.10→125.90 $R^2 = 0.9998$ $y = 4.96325e5x + 26362.25299$ number of data points = 7	<u>transition</u> 209.10→90.00 $R^2 = 0.9998$ $y = 1.03122e5x + 2787.90438$ number of data points = 7
Assessment of matrix effects is presented	yes		yes	
Limit of determination (LOD)	0.0015 mg/kg		0.0015 mg/kg	
Limit of quantification (LOQ)	0.005 mg/kg		0.005 mg/kg	

Conclusion

The analytical method for determining the residues of acetamiprid and acetamiprid-N-desmethyl in honey that was reproduced meets the criteria of SANTE/2020/12830 Rev. 2, 14 February 2023 document in terms of precision, accuracy and uncertainty.

The method was validated over the concentration range of 0.005 - 0.05 mg/kg (µg/g) for acetamiprid and acetamiprid-N-desmethyl. Limit of detection was established at 0.0015 mg/kg.

The method acceptable as ILV for the primary method.

A 1.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

A 1.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

Please refer to point A 2.1.1.2.4 (Honey).

A 1.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted.

A 1.1.2.7 A.2.A.9 Other Studies/ Information

Not relevant.