REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: ASA-01

Product name(s): **VIARES**

Chemical active substance:

Acetamiprid, 300 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: XXXX

Submission date: March 2024

Evaluation date: May 2025

MS Finalisation date: July 2025

Version history

When	What
May 2025	zRMS assessment
July 2025	Final RR updated with supplementary data provided by the applicant in the document: "Description of validation of analytical method for ASA-01 (Acetamiprid 300 g/L) SC for determination of acetamiprid"; Knapik I., 2025

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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 in the plant protection product.

Noticed data gaps are:

- none

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions. The dRR was not rewritten. zRMS comments are on grey background.
In the context of the authorisation no data gaps were found.

Commodity/crop	Supported/ Not supported
Oilseed rape	Supported
Pome fruits	Supported
~~Apple (incl. wild apple)~~	
~~Pear (incl. Chinese pear)~~	
~~Quince~~	
~~Medlar~~	

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of Evaluator:	The presented below analytical method has been validated according to EU Guidance SANCO/3030/99 rev.5. The method is acceptable for determination of acetamiprid in the formulation ASA-01.

Reference:	5.1.1/01
Report	FINAL REPORT. Determination of physicochemical properties. Test item: ASA-01 (ACETAMIPRID 300 g/L) SC, Knapik I., 2021, Report no. ICB/45/2021 Updated with "Description of validation of analytical method for ASA-01 (Acetamiprid 300 g/L) SC for determination of acetamiprid"; Knapik I., 2025
Guideline(s):	Yes, SANCO/3030/99 rev.5 (22/03/19)

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Determination of the content of acetamiprid was conducted according to own validated analytical method SPB/217. Content of acetamiprid at a level of 300 g/L in the test item was accordingly determined by liquid chromatography with diode array detection (HPLC-DAD).

Equipment and materials

- acetonitrile (ACN) HPLC, (VWR),
- 85% phosphoric acid (Merck),
- water ultra-purification unit HLP 5UV, W/10 (Hydrolab, Poland),
- placebo,
- acetamiprid standard; LGC; batch G1050293,
- standard stock solution of acetamiprid in ACN/H2O (1:1) mixture,
- working standard solutions of acetamiprid in ACN/H2O (1:1) mixture,
- analytical balance – accuracy 0.0001 g, WP/16 (Ohaus, Switzerland),
- liquid chromatograph with diode array detection, WP/42 (Shimadzu, Japan),
- chromatography column type C18, 150 mm x 4.6 mm; 5 µm (Phenomenex, Luna Omega Polar), K/15/HPLC
- ultrasonic bath, W/29 (Branson 200, Taiwan),
- chromatographic vials 1.5 mL with septa buthyl/Teflon,
- volumetric flask A class 10 mL,
- measuring syringe 500 µL.

Chromatography parameters

- Column: C18, 150 mm x 4.6 mm; 5 µm (Phenomenex, Luna Omega Polar)
- Analysis time: 8 min
- Flow: 1.4 mL/min
- Column temperature: 25°C
- Mobile phase: A – Acetonitrile, B – Water 0.1% H_3PO_4
- Isocratic program: phase A – 30%, phase B – 70%
- Injection: 10 µl
- Detector DAD: 246 nm

Method validation

For validation method for determination of acetamiprid in the test item were prepared two series of measurements, one without addition standard and second with addition standard. Calibration curve of active ingredient was prepared to determine the linear range.
To determine the average content of active ingredient (validation level 100%) and precision, was prepared series of measurements (n=5) of the test item without standard addition. Approximately 80 mg of test item was weighed out into 10 mL volumetric flask and was filled up to the mark with ACN/H_2O (1:1) mixture. The content of the flask was sonicated for 5 minutes. 400 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with ACN/H_2O (1:1) mixture. Then final solution was placed in chromatographic vial and analysed.
To determine recovery was prepared series of measurements (n=5) of the test item with standard addition. Approximately 80 mg of test item was weighed out into 10 mL volumetric flask. Then 800 µL 7.002 mg/mL stock solution of acetamiprid, was added and volumetric flask was filled up to the mark with ACN/H_2O (1:1) mixture. The content of the flask was sonicated for 5 minutes. 400 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with ACN/H_2O (1:1) mixture. Then final solution was placed in chromatographic vial and analysed.

The average recovery and precision for validation level LOQ, was determined by prepared series of measurements (n=5) of placebo with standard addition. Approximately 80 mg of placebo was weighed out into 10 mL volumetric flask. Then 37 µL 7.002 mg/mL was added and volumetric flask was filled up to the mark with ACN/H_2O (1:1) mixture. The content of the flask was sonicated for 5 minutes. 400 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with ACN/H2O (1:1) mixture. Then final solution was placed in chromatographic vial and analysed.
The average recovery and precision for validation level ULOQ, was determined by prepared series of measurements (n=5) of placebo with standard addition. Approximately 80 mg of placebo was weighed out into 10 mL volumetric flask. Then 700 µL 7.002 mg/mL was added and volumetric flask was filled up to the mark with ACN/H_2O (1:1) mixture. The content of the flask was sonicated for 5 minutes. 400 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with ACN/H_2O (1:1) mixture. The sample was 25-fold dilution to fit the linear range of active ingredients. Then final solution was placed in chromatographic vial and analysed.

Linearity

In order to check the linearity of acetamiprid, calibration curve was prepared using standard solutions with concentrations contained in table below. A graph of the peak area to the concentration of acetamiprid was plotted. The resulting curve is linear in the tested concentrations. Linearity range of acetamiprid is from 1.036 to 205.4 µg/mL, corresponding to ~~0.32-64.0%~~ 1.15-230%. Correlation coefficient R^2 is 0.9998944 and the linear regression is described by equation: $f(x)=2.64510 \cdot 10^{-5}x - 0.0231820$.

Concentration of standard solution [mg/mL]	Volume of standard solution [µL]	Flask volume [mL]	Concentration of working solutions [µg/mL]	Calibration level
1.027	10	10	1.027	Cal 1
1.027	100	10	10.27	Cal 2
1.027	500	10	51.35	Cal 3
1.027	1000	10	102.7	Cal 4
1.027	2000	10	205.4	Cal 5

Specificity

Specificity of the method was evaluated based on the analysis of chromatograms for placebo and samples of the test item against chromatograms of acetamiprid standard and peak purity. Analysis showed no overlapping of determined ingredient signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substance acetamiprid in plant protection product ASA-01

	acetamiprid
Author(s), year	Knapik I., 2021
Principle of method	HPLC-DAD
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	Linearity range of acetamiprid is from 1.036 to 205.4 µg/mL (corresponding to ~~0.32-64.0%~~ 1.15-230% of nominal content in the sample) Correlation coefficient: R^2 = 0. 9998944 Required: $R^2 \geq 0.99$ $f(x)=2.64510 \cdot 10{-}5x - 0.0231820$
Precision – Repeatability Mean n = 5 (%RSD)	Mean content = 27.9% w/w H_r = 0.63 Required: $H_r \leq 1.0$

	acetamiprid
	RSD = 1.02% Required: RSD ≤ 1.62% The obtained result is acceptable.
Accuracy **n = 5** **(% Recovery)**	Total recovery Standard addition 25.10%: Recovery: 97.12-101.93%; mean recovery:100.1% Required: 97-103% Marginal recovery Standard addition 0.32%: Recovery: 99.3-101.7%; mean recovery:100.1% Required: 80-120% Standard addition 6.12%: Recovery: 99.7-101.8%; mean recovery:100.7% Required: 90-110% The obtained results are acceptable.
Interference/ Specificity	Analysis showed no overlapping of determined ingredient signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled Based on chromatograms provided in the study report: no interference on test item response from co-formulants/reagents in the retention time region of the active from control samples was found.
Comment	No comments. The analytical method is fully validated

Conclusion

The HPLC-DAD method, used to quantify active substance acetamiprid in ASA-01 product was fully validated. The method fulfils requirements of SANCO/3030/99 rev.5.

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

There are no relevant impurities in plant protection product ASA-01.

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

No analytical method is required.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

CIPAC method is available for the determination of Acetamiprid (CIPAC method 649/SP/M/3).

5.2.2 Methods for the determination of residues (KCP 5.1.2)

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

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**
APP medium *Raphidocelis subcapitata* (Ecotoxicology)	Primary & confirmatory	0.1 mg/L	HPLC-DAD	Janota D., 2020 / Report No. W/50/19
Soil *Hypoaspis* (*Geolaelaps*) *aculeifer* (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC-DAD	Wołany M., 2020 / Report No. G/56/19
Water *Apis mellifera* L. (Larval Toxicity Test, Single Exposure) (Ecotoxicology)	Primary & confirmatory	0.05 mg/L	HPLC-DAD	Kulec-Płoszczyca E., 2020 / Report No. B/64/19
Water *Apis mellifera* L. (Larval Toxicity Test, Repeated Exposure) (Ecotoxicology)	Primary & confirmatory	0.06 mg/L	HPLC-DAD	Kulec-Płoszczyca E., 2021 / Report No. B/65/19
50% sucrose solution *Apis mellifera* L. (Chronic Oral Toxicity Test) (Ecotoxicology)	Primary & confirmatory	0.5 mg/kg	HPLC-DAD	Kulec-Płoszczyca E., 2021 / Report No. B/63/19
50% sucrose solution *Bombus* spp. (Acute Oral Toxicity Test) (Ecotoxicology)	Primary & confirmatory	0.2 mg/kg	HPLC-DAD	Myrczek, E., 2020 / Report No. B/66/19
Distilled water with 1% of surfactant *Bombus* spp. (Acute Contact Toxicity Test) (Ecotoxicology)	Primary & confirmatory	0.1 mg/L	HPLC-DAD	Myrczek, E., 2020 / Report No. B/67/19
Water Terrestrial Plant Test: Vegetative Vigour Test (Ecotoxicology)	Primary & confirmatory	0.1 mg/L	HPLC-DAD	Wołany M., 2020 / Report No. G/58/19
Water Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test (Ecotoxicology)	Primary & confirmatory	0.1 mg/L	HPLC-DAD	Pieczka P., 2020 / Report No. G/59/19
Soil *Eisenia andrei* (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC-DAD	Pieczka P., 2020 / Report No. G/54/19
Elendt M7 medium *Daphnia magna*	Primary & confirmatory	0.0005 mg/L	HPLC-DAD	Kacperek-Karetta G., 2023 / Report No. W-44-

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**
(Ecotoxicology)				22
Apple - fruit (Residues)	Primary & confirmatory	0.01 mg/kg	HPLC-MS/MS	Bagnall J., 2022 / Report No. JBL-20-45212
Oilseed rape - seed and plant (Residues)	Primary & confirmatory	0.01 mg/kg (whole plant & seeds)	HPLC-MS/MS	Domingo S., 2023 / Report No. SDO-20-45215
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	Wańczyk K., 2023 / Report No. 23SGS28
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	Wańczyk K., 2023 / Report No. 23SGS27

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	LC-MS/MS	Wańczyk K., 2023 / Report No. 23SGS26

		(IM-2-1) expressed as acetamiprid		

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substance in the plant protection product shall be submitted. The method already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

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

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

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

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Acetamiprid	0.01 mg/kg	Reg. (EU) 2019/88
Plant, high acid content		0.01 mg/kg	Reg. (EU) 2019/88
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	Reg. (EU) 2019/88
Plant, high oil content		0.01 mg/kg	Reg. (EU) 2019/88
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg	Reg. (EU) 2019/88
Muscle	N-desmethyl-acetamiprid (IM-2-1), expressed as acetamiprid	0.02 mg/kg	Reg. (EU) 2019/88
Milk		0.2 mg/kg	Reg. (EU) 2019/88
Eggs		0.02 mg/kg	Reg. (EU) 2019/88
Fat		0.02 mg/kg	Reg. (EU) 2019/88
Liver, kidney		0.02 mg/kg	Reg. (EU) 2019/88
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	*Chironomus riparius* EFSA Journal 2016;14(11):4610
Air	Acetamiprid	7.5 µg/m^3 AOEL sys: 0.025 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
		bw/d	
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

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

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

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 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.		

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

Table 5.3-3: Statement on extraction efficiency

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

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid in animal matrices is given in the following tables.

Table 5.3-4: Validated methods for food and feed of animal origin

Component of residue definition: *N*-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**
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)	Not 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	Not required.		

Component of residue definition: *N*-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**
	(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)	Not 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)	Not 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)	Not required.		
Component of residue definition: sum of acetamiprid and N-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**
Honey	Primary	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	Wańczyk K., 2023 / Report No. 23SGS26
	ILV	-	-	-

Component of residue definition: *N*-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**
	Confirmatory (if required)	Provided with primary method.		

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

Table 5.3-5: Statement on extraction efficiency

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

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid in soil is given in the following tables.

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	Täufer, A. and Weber, H., 2010, Study No. RD-02062N , EU agreed, Netherlands, RAR, 2015
Confirmatory	Not required.		

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

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid in surface and drinking water is given in the following tables.

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

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**
	ILV	0.1 µg/L (acetamiprid)	HPLC-MS/MS	Senciuc, M., 2014a, Study No. RD-01951, EU agreed, Netherlands, RAR, 2015 to which is equivalent Eichler M., Hermann S., 2018, 133112101 (unprotected study from DML Los Ovados 200 SE)
	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 to which is equivalent Eichler M., 2018, 133141101 (unprotected study from DML Los Ovados 200 SE)
	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.		

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

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid in air is given in the following tables.

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

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

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

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

An overview on the acceptable methods and possible data gaps for analysis of acetamiprid in body fluids and tissues is given in the following table.

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) 0.01 mg/kg (muscle, liver)	LC-MS/MS	Kenji Miya, 2003, Study No.: NCAS 03-235 / Ellas, DAR Addendum3, January 2004
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 2.

No residue definition was provided for body fluids and tissues (EFSA Journal 2016;14(11):4610). The applicant provided the analytical methods for determination of acetamiprid in body fluids and tissues which were evaluated as part of the EU review and is suitable for determination of acetamiprid residues in body fluids and tissues.

5.3.2.8 Other studies/ information

Not required.

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.1/01	Knapik I.	2021	Determination of physicochemical properties. Test item: ASA-01 (ACETAMIPRID 300 g/L) SC Report no. ICB/45/2021 Updated with "Description of validation of analytical method for ASA-01 (Acetamiprid 300 g/L) SC for determination of acetamiprid"; Knapik I., 2025 ICB Pharma GLP Unpublished	N	XXXX
KCP 5.1/01 (filed as KCP 10.2.1.3/01)	Janota D.	2020	ASA-01 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test Report No. W/50/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Janota D.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*) Growth inhibition test Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna, Poland Study code: W/50/19 GLP: Y Published: N		
KCP 5.1/02 (filed as KCP 10.4.2.1/01)	Wołany M.	2020	ASA-01 Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil Report No. G/56/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Czarnynoga M.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01: Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil Report No. G/56/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		

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 5.1/03 (filled as KCP 10.3.1.3/01)	Kulec-Płoszczyca E.	2020	ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Single Exposure Report No. B/64/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Kulec-Płoszczyca E.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Single Exposure Report No. B/64/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1/04 (filled as KCP 10.3.1.4/01)	Kulec-Płoszczyca E.	2021	ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Repeated Exposure Report No. B/65/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
KCP 5.1/05 (filled as KCP 10.3.1.2/01)	Kulec-Płoszczyca E.	2020	ASA-01 Honeybees (*Apis mellifera* L.), Chronic Oral Toxicity Test Report No. B/63/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
KCP 5.1/06 (filled as KCP 10.3.1.1.1/02)	Myrczek E.	2020	ASA-01 Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test Report No. B/66/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Myrczek E.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test Report No. B/66/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
	Myrczek E.	2020	ASA-01 Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test Report No. B/67/19	N	XXXX

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 5.1/07 (filled as KCP 10.3.1.1.2/02)			Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
	Myrczek E.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test Report No. B/67/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1/08 (filed as KCP 10.6.2/02)	Wołany M.	2020	ASA-01 Terrestrial Plant Test: Vegetative Vigour Test Report No. G/58/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Czarnynoga M.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Terrestrial Plant Test: Vegetative Vigour Test Report No. G/58/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1/09 (filed as KCP 10.6.2/01)	Pieczka P.	2020	ASA-01 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test Report No. G/59/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
	Pieczka P.	2021	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test Report No. G/59/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1/10 (filed as KCP 10.4.1.1/01)	Pieczka P.	2020	ASA-01 Earthworm Reproduction Test (*Eisenia andrei*) Test Report No. G/54/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna	N	XXXX

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		
	Pieczka P.	2020	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Earthworm reproduction test (*Eisenia andrei*) Report No. G/54/19 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished		
KCP 5.1/11 (filed as KCP 10.2.1.2/01)	Kacperek-Karetta G.	2023	ASA-01 *Daphnia magna*, Acute Immobilisation Test Report No. W-44-22 Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Branch Pszczyna GLP Unpublished	N	XXXX
KCP 5.1/12	Bagnall J.	2022	Acetamiprid – Residue Study on Apples in Northern Europe – 2020 Report No. JBL-20-45212 (ANALYTICAL PHASE REPORT GLP-STUDY-20-40) STAPHYT Ltd. GLP Unpublished	N	XXXX
KCP 5.1/13	Domingo S.	2023	Acetamiprid – Residue Study on winter oilseed rape in Northern Europe – 2020 Report No. SDO-20-45215 (ANALYTICAL PHASE REPORT GLP-STUDY-20-26) STAPHYT Ltd. GLP Unpublished	N	XXXX
KCP 5.1/14	Niewelt-Stasiak S.	2023	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in apple Report No. VAL/15/2023 SGS Polska Sp. z o. o. GLP Unpublished	N	XXXX
KCP 5.1/15	Niewelt-Stasiak S.	2023	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant) Report No. VAL/14/2023 SGS Polska Sp. z o. o. GLP	N	XXXX

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 5.1/16	Niewelt-Stasiak S.	2023	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in honey Report No. VAL/13/2023 SGS Polska Sp. z o. o. GLP Unpublished	N	XXXX

* XXXX

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 Upublished	N	XXXX
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 Agroscience Services GLP Unpublished	N	XXXX
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 Agroscience Services GLP	N	XXXX

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.	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	XXXX
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	XXXX
KCP 5.2	Täufer, 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 Unpublished	N	XXXX
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	XXXX
KCP 5.2	Matthias Eichler, Herrmann, S.	2018	Acetamiprid: Independent Laboratory Validation of an Analytical Method for the Determination in Drinking Water Study No. 133112101 ibacon GmbH Arheilger Weg 17 64380 Rossdorf Germany GLP: Yes Unpublished	N	XXXX
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 Agroscience Services, Germany,	N	XXXX

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, not published		
KCP 5.2	Dr. Matthias Eichler Silke Herrmann	2018	IM-1-5 (Metabolite of Acetamiprid): Independent Laboratory Validation of an Analytical Method for the Determination in Drinking Water Study No. 133141101 ibacon GmbH Arheilger Weg 17 64380 Rossdorf Germany GLP: Yes Unpublished	N	XXXX
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	XXXX
KCP 5.2	Kenji Miya	2003	Development and Validation of the Analytical Method for the Determination of Acetamiprid in Body Fluids and Tissues Nisso Chemical Analysis Cervice Co., Ltd. Nippon Soda Co., Ltd. Study No.: NCAS 03-235 GLP Unpublished	N	XXXX

** unprotected study from DML Los Ovados 200 SE

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for acetamiprid

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

A 2.1.1.1 Description of analytical methods for the determination of active substance and/or variant in the plant protection product

Please refer to the point 5.2.1.1.

A 2.1.1.2 Description of analytical methods used in ecotoxicological studies

A 2.1.1.2.1 HPLC - DAD detection (in APP medium)

A 2.1.1.2.1.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/01 (filed as KCP 10.2.1.3/01)
Report	ASA-01 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*), Growth inhibition test, Report No. W/50/19, Janota D., 2020
	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 *Raphidocelis subcapitata* SAG 61.81 (formerly *Pseudokirchneriella subcapitata*) Growth inhibition test, Report No. W/50/19, Janota D., 2021
Guideline(s):	SANCO/3029/99 rev.4.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The aim of the analytical part of the definitive test was determination of the test item concentration using validated liquid chromatographic method with DAD detection. The validated analytical method was performed according to SANCO/3029/99 rev. 4.
The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatographic System: Shimadzu Prominence-i liquid chromatograph with DAD LC-2030C 3D (Shimadzu Corpo-ration)
- Column: Gemini NX 3µ C18 100A, l = 150 mm, Ø = 4.6 mm
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid (35 : 65, v/v)
- Wavelength: 246 nm

- Oven temperature 35°C
- Flow Rate: 0.5 mL/min
- Injection volume: 20 µl

Sample preparation for the chemical determinations
Each sample in a volume of 5 mL was taken and diluted with mixture of acetonitrile and 0.05% orthophosphoric acid (30:70 v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
Working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0. 5.0, and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient of 0.9998325. The range of linearity of the analytical graph is from 0.01 µg/mL to 10.0 µg.

Specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control water samples and fortified samples. 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.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Precision
In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of acetamiprid in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.10	0.100	0.099	0.100	0.098	0.098	0.099	99.0	0.001	1.0
10.0	10.06	10.09	10.10	10.10	10.11	10.09	100.9	0.02	0.2

LoQ = 0.1 mg/L
LoD = 0.03 mg/L
SD – standard deviation
RSD – relative standard deviation

Limit of quantification and limit of detection
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

Limit of Quantification (LoQ) for acetamiprid is 0.1 mg/L and Limit of Detection (LoD) is 0.03 mg/L.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item ASA-01 in APP medium.

A 2.1.1.2.2 HPLC - DAD detection (in soil)

A 2.1.1.2.2.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/02 (filed as KCP 10.4.2.1/01)
Report	ASA-01 Predatory mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) reproduction test in soil, Report No. G/56/19, Wołany M., 2020 AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01: Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil; Czarnynoga M.; 2021; Study Code: G/56/19
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substance of ASA-01 in artificial soil was determined using the validated high performance liquid chromatographic method with DAD detection. The validated analytical method was performed according to SANCO/3029/99 rev.4.
Samples collected at the beginning, during (after one week) and at the end of the experiment were analysed. The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini-NX 3µ C18 100A, l=150 mm, Ø=4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% solution of orthophosphoric acid (35:65, v/v)
- Flow Rate: 0.50 mL/min
- Wavelength: 246 nm
- Oven temperature: 35°C
- Detection System: Diode Array Detector

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 : 0.05% ortho-phosphoric acid (30 : 70, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
The working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r2) of 0.9998325. The range of linearity of the analytical graph is from 0.01 µg/mL to 10.0 µg/mL.

Specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control artificial soil samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated artificial soil samples and then analysed. The results are presented in table below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for test item analysed in the test samples are presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/kg]	Determined concentration of acetamiprid in replicates [mg/kg]					Average [mg/kg]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.0000	0.0000	-	-	-	0.0000	-	0.0000	-
0.05	0.0426	0.0429	0.0486	0.0495	0.0484	0.0464	92.8	0.0034	7.2
5.0	4.7472	4.7261	4.7274	4.7327	4.7244	4.7316	94.6	0.0093	0.2

LoQ = 0.05 mg/L
LoD = 0.015 mg/L
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LOQ) for test item analysed in artificial soil is 0.05 mg/kg and the Limit of Detection (LOD) is 0.015 mg/kg.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in soil.

A 2.1.1.2.3 HPLC - DAD detection (in water)

A 2.1.1.2.3.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/03 (filled as KCP 10.3.1.3/01)
Report	ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Single Exposure, Kulec-Płoszczyca E., 2020, Report No. B/64/19

	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Single Exposure; Kulec-Płoszczyca E.; 2021; Report No. B/64/19
Guideline(s):	SANCO 3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substances of test item was chemically determined using the validated high performance liquid chromatographic method with DAD detection. The analytical method was developed for the determination of active substance of test item in matrix. The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatographic system: HPLC Shimadzu Prominence-*i* chromatograph with DAD
- Column: Luna 5µm C18 100A, l=250 mm, Ø=4.6 mm
- Mobile phase: acetonitrile for HPLC : 0.05% solution of ortho-phosphoric acid (40:60, v/v)
- Wavelength: 246 nm
- Oven temperature: 35°C
- Flow rate: 1.0 mL/min
- Injection volume: 20 µl
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample in a volume of 5-10 mL was diluted in ratio 1:1 with acetonitrile HPLC. The samples were diluted with mixture acetonitrile HPLC and 0.05% ortho-phosphoric acid (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity

The working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r^2) of 0.9999141. The range of linearity of the analytical graphs is from 0.02 µg/mL to 10.0 µg/mL.

Specificity

The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control water samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Precision

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Recovery level and precision of acetamiprid in fortified water samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of acetamiprid in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.05	0.0508	0.0510	0.0506	0.0502	0.0506	0.0506	101.2	0.0003	0.6
0.5	0.498	0.502	0.504	0.504	0.500	0.502	100.4	0.003	0.5

LoQ = 0.05 mg/L
LoD = 0.15 mg/L
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LoQ) for detected substance analysed in water is 0.05 mg/L and the Limit of Detection (LoD) is 0.015 mg/L.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in water.

A 2.1.1.2.4 HPLC - DAD detection (in water)

A 2.1.1.2.4.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/04 (filled as KCP 10.3.1.4/01)
Report	ASA-01 Honeybees (*Apis mellifera* L.), Larval Toxicity Test, Repeated Exposure, Kulec-Płoszczyca E., 2021, Report No. B/65/19
Guideline(s):	SANTE/2020/12830, Rev. 1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of 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 of acetamiprid and limit of quantification and detection of acetamiprid were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD

detection. Prior to analysis, the samples were diluted. The validated analytical method was performed according to SANTE/2020/12830, Rev. 1.

Chromatographic conditions

- Chromatograph: High Performance Liquid Chromatography (HPLC), Shimadzu, Prominence-*i* (Shimadzu Corporation Japan)
- Analytical Column: Luna 5µm C18(2) 100Å , l = 250 mm, Ø = 4.6 mm
- Oven temperature: 35°C
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% ortho-phosphoric acid (40:60, v/v)
- Flow Rate: 1.0 mL/min
- Wavelength: 246 nm
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample in a volume of 5 mL was diluted in ratio 1-1 with acetonitrile HPLC. The eluate was diluted with mixture 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.

Preparation of Fortified Sample

For validation experiments, 5 mL of untreated water were spiked with appropriate volumes of fortification solutions. The following fortification scheme was used.

Sample Type	Sample [mL]	Concentration of Spiking Solution of acetamiprid [µg/mL]	Volume of Spiking Solution [µL]	Level of Fortification [mg/L]
Control	5	-	-	0.00
Fortification (LOQ)	5	10	30	0.06
Fortification (10x LOQ)	5	100	30	0.6

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 i.e. 0.6 mg acetamiprid/L.
This was done to ensure the result fits within the range of the respective standard curve.

Validation of analytical method

Linearity

The stock solution of acetamiprid with a concentration of 1 mg/mL was prepared by weighting 10 mg analytical standards 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 was prepared by dilution of the stock solutions with acetonitrile for HPLC. Moreover, the working solutions of acetamiprid with a concentration of 10 µg/mL were prepared by dilution of the working solution at concentration of 100 µg/mL detected substance with acetonitrile for HPLC. Calibration and fortification solutions containing of acetamiprid were prepared by dilution of working solution at concentration 100 µg acetamiprid/mL in acetonitrile for HPLC. Further dilutions were conducted with mixture of acetonitrile for HPLC and deionized water (50:50, v/v) as exemplarily described in the table below.

Take solution [µg/mL]	Aliquot Volume [mL]	Dilute with solution to a final volume of [mL]	Final Concentration [µg/mL]

1000	1	10	100
100	1	10	10[1)]
100	0.5	10	5[1)]
10	3	10	3
10	2	10	2[1)]
10	1	10	1[1)]
5	1	10	0.5[1)]
3	1	10	0.3
2	1	10	0.2[1)]
1	1	10	0.1[1)]
0.5	1	10	0.05[1)]
0.3	1	10	0.03
0.2	1	10	0.02[1)]

[1)] Concentration level used for calibration.

Working solutions of acetamiprid at the concentrations of 0.02 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The range of the first linearity of the analytical graph of acetamiprid are from 0.02 µg/mL to 1 µg/mL. The ranges of calibration curve of acetamiprid is equivalent to range from 0.04 mg/L to 2.0 mg/L in water.
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.

Analyte	**Slope**	**Intercept**	**Coefficient r^2**
Acetamiprid	106575	50.8927	0.9998556

The range of the second linearity of the analytical graph of acetamiprid are from "0.5 µg/mL to 10 µg/mL. The ranges of calibration curve of acetamiprid is equivalent to range from 1.0 mg/L to 20 mg/L in water.
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.

Analyte	**Slope**	**Intercept**	**Coefficient r^2**
Acetamiprid	105225	829.651	0.9998877

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 in a residual plot of acetamiprid for range from 0.02 µg/mL to 1.0 µg/mL is presented Figure 2. The regression residuals is presented in a residual plot of acetamiprid for range from 0.5 µg/mL to 10 µg/mL

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 is 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.

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]).
The repeatability for detected substance analysed in the water is from 0.3% to 2.0%.
The precision is 2.0% at level 0.06 mg acetamiprid/L water and 0.3% at level 0.6 mg acetamiprid/L water.
The RSD for method is ≤ 20% per each level.

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. Recovery was reported as mean recovery ± relative standard deviation (RSD). Mean recoveries for each level is in the range 70-120%.
The mean recovery range for acetamiprid in water is from 94.3 ± 0.3% to 96.7 ± 2.0%. For analyte, the relative standard deviations (RSD) at each fortification levels were below 10%.

Matrix	Active substance	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
Water	acetamiprid	0.06	5	96.7	2.0
		0.6	5	94.3	0.3

Matrix Effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard at concentration 0.03 µg acetamiprid/mL prepared in solvent to at concentration 0.03 µg acetamiprid/mL prepared in blank matrix of water.
Matrix effect, expressed in % enhancement or suppression was evaluated according to the following equation:

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

Matrix effect for acetamiprid is -5.0 % and not exceed ±20%.

Limit of detection (LOD) and limit of quantification (LOQ)
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 120% with a relative standard deviation of preferably ≤ 20%).
The LoQ of analytical method 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 of analytical method is: 0.04 mg acetamiprid/L water and equivalent to the lowest calibration standard i.e. 0.02 µg acetamiprid/mL.

Stability
The stability of stock solution of acetamiprid was tested at concentrations 1000 mg acetamiprid/L. Data for stability were obtained after 0 day, 6 days and 21 days of storage at cool temperature i.e. 2°C – 8°C. Compared to the mean recoveries measured at 0 day, significant decline was not observed after 21 days.
The results are presented in table below.

Days of Storage	Storage temperature 2-8°C
	Stock solution of acetamiprid at concentration 1000 mg/L
	Mean recovery ± RSD[%]
0	105.3 ± 0.4
6	105.8 ± 0.9
21	103.8 ± 0.5

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in water.

A 2.1.1.2.5 HPLC - DAD detection (in 50% sucrose solution)

A 2.1.1.2.5.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/05 (filled as KCP 10.3.1.2/01)
Report	ASA-01 Honeybees (*Apis mellifera* L.), Chronic Oral Toxicity Test, Kulec-Płoszczyca E., 2020, Report No. B/63/19
Guideline(s):	SANCO 3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method was developed for the determination of acetamiprid in 50% sucrose solution. The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in process of the analytical method validation.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Luna 5µm C18 100A, l=250 mm, Ø=4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile : 0.05% solution of orthophosphoric acid (40:60, v/v)
- Flow Rate: 1.0 mL/min
- Wavelength: 246 nm
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
First, 1 g sucrose sample was weighted into a volumetric flask with a capacity of 10 mL and made up to 5 mL of solution 0.05% ortho-phosphoric acid. Next, the volume was made up to 10 ml with acetonitrile. The eluate was diluted with mixture acetonitrile and 0.05% orthophosphoric acid (50:50; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
The working solutions of acetamiprid at the concentrations of 0.02, 0.05, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r^2) of 0.9999141. The range of linearity of the analytical graphs range from 0.02 µg/mL to 10.0 µg/mL.

Specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control sucrose samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated sucrose samples and then analysed. The results are presented in table below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the test samples are presented in table below.

Recovery level of acetamiprid in fortified sucrose solution samples (n = 5)

Nominal concentration [mg/kg]	Determined concentration of acetamiprid in replicates [mg/kg]					Average [mg/kg]	Recovery [%]	SD [mg/kg]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.5	0.517	0.504	0.505	0.507	0.503	0.507	101.4	0.006	1.1
5.0	5.016	5.034	4.992	5.018	4.998	5.012	100.2	0.017	0.3

LoQ = 0.5 mg/kg
LoD = 0.15 mg/kg
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LoQ) for detected substance analysed in sucrose is 0.5 mg/kg and the Limit of Detection (LoD) is 0.15 mg/kg.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in sucrose solution.

A 2.1.1.2.6 HPLC - DAD detection (in 50% sucrose solution)

A 2.1.1.2.6.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/06 (filled as KCP 10.3.1.1.1/02)
Report	ASA-01 Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test, Myrczek E., 2020, Report No. B/66/19
	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Bumblebees (*Bombus* spp.), Acute Oral Toxicity Test; Myrczek E; 2021; Report No. B/66/19
Guideline(s):	SANCO 3029/99 rev. 4

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of ASA-01, in sucrose solution was determined as peak acetamiprid using the validated high performance liquid chromatographic method with DAD detection.
The validated analytical method was performed according to SANCO/3029/99 rev.4.
Fresh samples of working test item at concentration 5 g/L (corresponding with 4226.54 mg/kg sucrose; density of sucrose is 1.183 g/cm3), 0.3125 g/L (corresponding with 264.16 mg/kg sucrose; density of sucrose is 1.183 g/cm3), and the control at the initial test, were analysed.
The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini NX 3µ C18 100A, l = 150 mm, Ø = 4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile : 0.05% solution of ortho-phosphoric acid (35:65, v/v)
- Flow Rate: 0.5 mL/min
- Wavelength: 246 nm
- oven temperature: 35°C
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
First, 1 g sucrose sample was weighted into a volumetric flask with a capacity of 10 mL and added 7 mL of solution 0.05% ortho-phosphoric acid. Next, the volume was made up to 10 ml with acetonitrile. The eluate was diluted with mixture acetonitrile and 0.05% orthophosphoric acid (30:70; v/v), if necessary. An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
Working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0. 5.0, and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient of 0.9998325. The range of linearity of the analytical graph is from 0.01 µg/mL to 10.0 µg/mL.

Specificity
The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control sucrose samples and fortified samples. 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.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated sucrose samples and then analysed. The results are presented in tables below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the fortified samples is presented in table below.

Recovery level and precision acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/kg]	Determined concentration of acetamiprid in replicates [mg/kg]					Average [mg/kg]	Recovery [%]	SD [mg/kg]	RSD [%]
	1	2	3	4	5				
Control	0.00	0.0	-	-	-	0.0	-	0.0	-
0.20	0.2110	0.2120	0.1960	0.2100	0.2060	0.2070	103.5	0.0066	3.2
2.0	2.011	2.021	2.017	2.012	2.027	2.018	100.9	0.007	0.3

LoQ = 0.2 mg/kg
LoD = 0.06 mg/kg
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

Limit of Quantification (LoQ) for acetamiprid analysed in sucrose is 0.2 mg/kg and Limit of Detection (LoD) is 0.06 mg/kg.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item ASA-01 in sucrose solution.

A 2.1.1.2.7 HPLC - DAD detection (in distilled water with 1% of surfactant)

A 2.1.1.2.7.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/07 (filled as KCP 10.3.1.1.2/02)
Report	ASA-01 Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test, Myrczek E., 2020, Report No. B/67/19 AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Bumblebees (*Bombus* spp.), Acute Contact Toxicity Test; Myrczek E; 2021; Report No. B/67/19
Guideline(s):	SANCO 3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of ASA-01 in water solution was determined as peak acetamiprid using the validated high performance liquid chromatographic method with DAD detection.
The validated analytical method was performed according to SANCO/3029/99 rev.4.

Fresh samples of working test item at concentration 100 g/L, and the control at the initial test, were analysed The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini NX 3µ C18 100A, l = 150 mm, Ø = 4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% solution of ortho-phosphoric acid (35:65, v/v)
- Flow Rate: 0.5 mL/min
- Wavelength: 246 nm
- Oven temperature 35°C
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

1-10 mL water sample was diluted with mixture acetonitrile and 0.05% orthophosphoric acid (30:70; v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity

Working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0. 5.0, and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient of 0.9998325. The range of linearity of the analytical graph is from 0.01 µg/mL to 10.0 µg/mL.

Specificity

The analytical method specificity was estimated on the basic of the analysis of the chromatograms obtained for the control water samples and fortified samples. 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.

Extraction recovery level

In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Precision

Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for detected substance analysed in the fortified samples is presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of acetamiprid in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.1	0.100	0.099	0.100	0.098	0.098	0.099	99.0	0.001	1.0
10.0	10.06	10.09	10.10	10.10	10.11	10.09	100.9	0.02	0.2

LoQ = 0.1 mg/L
LoD = 0.03 mg/L
SD – standard deviation
RSD – relative standard deviation

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

Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤

20%).

Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

Limit of Quantification (LoQ) for acetamiprid analysed in water is 0.1 mg/L and Limit of Detection (LoD) is 0.03 mg/L.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item ASA-01 in distilled water.

A 2.1.1.2.8 HPLC - DAD detection (in water)

A 2.1.1.2.8.1 Method validation

Comments of Evaluator:	The method validation has been accepted (by which the concentration of acetamiprid in water was determined).

Reference:	KCP 5.1/08 (filed as KCP 10.6.2/02)
Report	ASA-01 Terrestrial Plant Test: Vegetative Vigour Test, Wołany M., 2020, Report No. G/58/19 AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Terrestrial Plant Test: Vegetative Vigour Test; Czarnynoga M.; 2021; Report No. G/58/19
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substance of ASA-01 in test solution was determined using the validated high performance liquid chromatographic method with DAD detection. The validated analytical method was performed according to SANCO/3029/99 rev.4.
The aim of analytical measurements of the study was to verify the concentration of the test item at the doses of test solution and the control (i.e. 25.4, 50.7, 101.3, 202.1, 405.0 mL of the test item/ha).
The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini-NX 3µ C18 100A, l=150 mm, Ø=4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% solution of orthophosphoric acid (35:65, v/v)
- Flow Rate: 0.50 mL/min
- Wavelength: 246 nm
- Oven temperature: 35°C
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
Each sample in a volume of 1-5 ml was diluted in mixture of acetonitrile and 0.05% ortho-phosphoric acid (30 : 70, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
The working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r^2) of 0.9998325. The range of linearity of the analytical graphs range from 0.01 µg/mL to 10.0 µg/mL.

Specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control water samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed. The results are presented in table below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for test item analysed in the test samples are presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of acetamiprid in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.1	0.100	0.099	0.100	0.098	0.098	0.099	99.0	0.001	1.0
10.0	10.06	10.09	10.10	10.10	10.11	10.09	100.9	0.02	0.2

LoQ = 0.1 mg/L
LoD = 0.03 mg/L
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LOQ) for test item analysed in water is 0.1 mg/L and the Limit of Detection (LOD) is 0.03 mg/L.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in water.

A 2.1.1.2.9 HPLC - DAD detection (in water)

A 2.1.1.2.9.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/09 (filed as KCP 10.6.2/01)
Report	ASA-01 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, Pieczka P., 2020, Report No. G/59/19 AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test; Pieczka P.; 2021; Report No. G/59/19
Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substance of ASA-01 in test solution was determined using the validated high performance liquid chromatographic method with DAD detection. The validated analytical method was performed according to SANCO/3029/99 rev.4.
The aim of analytical measurements of the study was to verify the concentration of the test item at the doses of test solution and the control (i.e. 25.4, 50.7, 101.3, 202.1, 405.0 mL of the test item/ha).
The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini-NX 3µ C18 100A, l=150 mm, Ø=4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC : 0.05% solution of orthophosphoric acid (35:65, v/v)
- Flow Rate: 0.50 mL/min
- Wave length: 246 nm
- Oven temperature: 35°C
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis
Each sample in a volume of 1-5 ml was diluted in mixture of acetonitrile and 0.05% ortho-phosphoric acid (30 : 70, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
The working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r^2) of 0.9998325. The range of linearity of the analytical graph is from 0.01 µg/mL to 10.0 µg/mL.

Specificity

The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control water samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated water samples and then analysed using the method described above. The results are presented in table below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for test item analysed in the test samples are presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of acetamiprid in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.000	0.000	-	-	-	0.000	-	0.000	-
0.1	0.100	0.099	0.100	0.098	0.098	0.099	99.0	0.001	1.0
10.0	10.06	10.09	10.10	10.10	10.11	10.09	100.9	0.02	0.2

LoQ = 0.1 mg/L
LoD = 0.03 mg/L
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LOQ) for test item analysed in water is 0.1 mg/L and the Limit of Detection (LOD) is 0.03 mg/L.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substances of the test item ASA-01 in water.

A 2.1.1.2.10 HPLC - DAD detection (in soil)

A 2.1.1.2.10.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/10 (filed as KCP 10.4.1.1/01)
Report	ASA-01 Earthworm Reproduction Test (*Eisenia andrei*) Test, Pieczka P., 2020, Report No. G/54/19
	AMENDMENT NO. 1 TO THE FINAL REPORT ASA-01 Earthworm reproduction test (*Eisenia andrei*); Pieczka P.; 2021; Report No. G/54/19

Guideline(s):	SANCO/3029/99 rev.4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substance of ASA-01 in artificial soil was determined using the validated high performance liquid chromatographic method with DAD detection. The validated analytical method was performed according to SANCO/3029/99 rev.4.
Samples collected at the beginning, during (after four weeks) and at the end of the experiment were analysed.
The linearity of response of the analytical method, its specificity, precision, recovery, limit of quantification and detection were assessed in the process of the analytical method validation.

Chromatographic conditions

- Chromatograph: Shimadzu Prominence-*i* liquid chromatograph with DAD
- Analytical Column: Gemini-NX 3µ C18 100A, l=150 mm, Ø=4.6 mm
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile for HPLC and 0.05% solution of orthophosphoric acid (35:65, v/v)
- Flow Rate: 0.50 mL/min
- Wavelength: 246 nm
- Oven temperature: 35°C
- Detection System: Diode Array Detector

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 and 0.05% ortho-phosphoric acid (30 : 70, v/v). An aliquot of the final volume was transferred into a HPLC vial for further quantification using HPLC-DAD.

Validation of analytical method

Linearity
The working solutions of acetamiprid at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were injected successively to the chromatographic column and the chromatograms were recorded. The standard curve (peak area versus quantity of the standard) is linear with coefficient (r^2) of 0.9998325. The range of linearity of the analytical graphs range from 0.01 µg/mL to 10.0 µg/mL.

Specificity
The analytical method specificity was estimated on the basis of the analysis of the chromatograms obtained for the control artificial soil samples and fortified samples. Considering the results of the analysis, no signal of detected substance was overlapping with the matrix signal of the control samples under the experimental conditions. Therefore, the specificity of the method was demonstrated.

Extraction recovery level
In order to study the recovery level, the solution of the detected substance was added to non-treated artificial soil samples and then analysed. The results are presented in table below.

Precision
Precision of a particular method is defined as its repeatability (RSD – relative standard deviation [%]). The repeatability for test item analysed in the test samples are presented in table below.

Recovery level and precision of acetamiprid in fortified samples (n = 5)

Nominal concentration [mg/kg]	Determined concentration of acetamiprid in replicates [mg/kg]					Average [mg/kg]	Recovery [%]	SD [mg/kg]	RSD [%]
	1	2	3	4	5				
Control	0.0000	0.0000	-	-	-	0.0000	-	0.0000	-
0.05	0.0480	0.0469	0.0493	0.0461	0.0464	0.0473	94.7	0.0013	2.77
5.0	4.513	4.510	4.413	4.427	4.417	4.456	89.1	0.051	1.15

LoQ = 0.05 mg/kg
LoD = 0.015 mg/kg
SD – standard deviation
RSD – relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)
The Limit of Quantification was estimated as the lowest concentration of a detected substance at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%).

The Limit of Detection was estimated as the lowest concentration of a detected substance that the analytical procedure can reliably differentiate from the background noise.

The Limit of Quantification (LOQ) for test item analysed in artificial soil is 0.05 mg/kg and the Limit of Detection (LOD) is 0.015 mg/kg.

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item ASA-01 in soil.

A 2.1.1.2.11 HPLC - DAD detection (in Elendt M7 medium)

A 2.1.1.2.11.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/11 (filed as KCP 10.2.1.2/01)
Report	ASA-01 *Daphnia magna*, Acute Immobilisation Test, Kacperek-Karetta G., 2023, Report No. W-44-22
Guideline(s):	SANTE/2020/12830, Rev. 2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The concentration of active substance of test item was chemically determined using the validated high performance liquid chromatographic method with DAD detection. The analytical method was developed for the determination of active substance of test item in matrix. The range of linearity of the analytical graph, the regression residual (di), selectivity and specificity, precision, accuracy, matrix effect, stock solution stability and limit of quantification and detection were determined.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD detection. The validation of analytical method was performed according to SANTE/2020/12830, Rev. 2.

Chromatographic conditions

- Chromatograph: Shimadzu, Prominence (Shimadzu Corporation Japan)
- Chromatographic System: High Performance Liquid Chromatography (HPLC)
- Analytical Column: Luna Omega 5 µm PS C18 100Å
- Injection Volume: 20 µl
- Mobile Phase: acetonitrile HPLC : ortho-phosphoric acid solution 0.05 % (50 : 50, v/v)
- Flow Rate: 0.70 mL/min
- Wavelength: 246 nm
- Oven temperature: 35°C
- Detection System: Diode Array Detector

Sample preparation for the chromatographic analysis

Each sample of 100 mL volume was applied to ENVI-18 (3 mL, 500 mg) column conditioned previously by sequential washing triple with 5 mL of methanol pure p.a., twice with 5 mL of deionized water. Following the sample introduction the column was dried for 5 minutes by vacuum. The part of sample with affinity to the column was eluted triple with 5 mL of methanol pure p.a. Eluate was evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in mixture of acetonitrile for HPLC and deionized water (50:50; v/v) applied to chromatographic column.

Validation of analytical method

Linearity

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

Analyte	Working solution concentrations [mg/L]	range of linearity of calibration curve [mg/L]	equivalent calibration range of linearity [mg/L Elendt M7 medium]
acetamiprid	0.01, 0.05, 0.1, 0.5 and 1.0	0.01 – 1.0	0.0001 – 0.01

The standard curve of acetamiprid (peak area versus quantity of the standard) is linear.
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 linearity was given in µg/mL (equal to mg/L).

range of linearity of calibration curve [mg/L]	Analyte	Slope	Intercept	Coefficient r^2
0.01 – 1.0	acetamiprid	178538	-67.2541	0.9992039

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

Selectivity and specificity

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

Precision

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

Accuracy

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

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

Analyte	Fortification Level [mg/L]	Number of Replicates	Mean Recovery [%]	RSD [%]
acetamiprid	0.0005	5	81.0	2.0
	0.005	5	95.0	0.4

In order to study the recovery level, the solution of the detected substance was added to non-treated control sample and then analysed.

Matrix Effect

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

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

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

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

Matrix	Analyte	Concentration [mg/L]	Matrix effect [%]
Elendt M7 medium	acetamiprid	0.05	7.2

Final extract stability

Final extract stability was not determined. The final extracts were analysed within 24 h.

Stock solution stability

Stock solution stability was determined accomplished by chromatographic method with DAD detection. 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, 31, 58, 71, 84, 85 and 112 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 observed after 85 days. The mean recoveries for stock solution does not differ by more than 10%. The results are presented in Table below.

Days of Storage	Average concentration [mg/L]	RSD [%]	Recovery [%]	Decrease in relation to the initial concentration [%]
0	994.1	0.3	99.4	-
31	1044.5	0.3	104.5	-5.1
58	1057.9	0.4	105.8	-6.4

71	1078.9	0.4	107.9	-8.5
84	1086.3	0.4	108.6	-9.3
85	1087.8	0.3	108.8	-9.4
112	1157.1	0.5	115.7	-16.4

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 ≤ 20%).
The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample.
Limit of quantification (LOQ) and a limit of detection (LOD) are presented in the table below.

Analyte	**matrix**	**LOQ [mg/L]**	**LOD [mg/L]**
acetamiprid	Elendt M7 medium	0.0005	0.0001

Conclusion

The method was fully validated. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance of the test item ASA-01 in Elendt M7 medium.

A 2.1.1.3 Description of analytical methods used in residue studies

A 2.1.1.3.1 HPLC-MS/MS (in apple fruit)

A 2.1.1.3.1.1 Method validation

Comments of Evaluator:	The method validation has been accepted.

Reference:	KCP 5.1/12
Report	Acetamiprid – Residue Study on Apples in Northern Europe – 2020, Report No. JBL-20-45212 (ANALYTICAL PHASE REPORT GLP-STUDY-20-40), Bagnall J., 2022
Guideline(s):	SANCO 825/00 rev. 8.1 and SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of this analytical phase is to determine the residue of acetamiprid on apples sample coming from the Field Phase of the Study JBL-20-45212 (Test Facility: Staphyt Ltd., Study Director: Jamie Bagnall). The analytical determinations were carried out using a HPLC-MS/MS method, validated during the analytical phase according to SANCO 825/00 rev. 8.1 and SANCO/3029/99 rev. 4 guidelines.
The evaluated validation parameters were linearity, recovery and precision, limit of quantification and detection, selectivity, confirmation, matrix effect and stability.

Chromatographic conditions

- System operating: HPLC-MS/MS
- Instrument: Agilent HPLC 1290 Infinity II + Agilent spectrometer 6470A Triple Quad
- Column: Agilent Zorbax RRHD Clirse Plus C18/1.9 µm, 2.1x50 mm
- Column temperature: 40°C
- Flow: 0.600 mL/min
- Injection volume: 2.50 µL
- Mobile phase A: 100% LC-MS grade water + 0.1 % of Formic Acid
- Mobile phase B: 100% methanol UPLC grade + 0.1% of Formic Acid
- Elution:

Time (min)	%A	%B
0.50	95.0	5.00
2.50	50.0	50.0
3.00	0.0	100.0

- Divert valve: waste from 0 to 1.5 minutes, from 1.5 to 3 MS than waste until end of the run
- Stop time: 6 minutes
- Source type: ESI
- Gas temperature: 350°C
- Gas flow (L/min): 11
- Nebulizer (psi): 60
- Sheath gas heater: 400°C
- Sheath gas flow (L/min): 12
- Capillary: 3000 V
- Vcharging: 0
- Acquiring mode: ESI positive, MRM (multi reaction monitoring.

Samples preparation

5 g aliquots of the homogenised sample were weighed in a 50 mL screw capped centrifuge PE test tube. 20 mL of acetonitrile and 5 mL of water were added to the sample. The samples were thoroughly shaken for 2 minutes. 4 g of NaCl were added to the sample, the sample was further shaken for 1 minute and then centrifuged at 5000 RPM for 5 minutes. An aliquot of the final extract (1.5 mL) was transferred in a HPLC glass vial for the final determination.
All samples were analysed the same day of extraction.

Validation of analytical method

Linearity

Acetamiprid linearity was checked in the range 0.84 – 104.80 µg/L (corresponding to 0.0034 – 0.492 mg/kg on sample). 5 different concentration levels (single injection) were tested. The linearity equation and R^2 correlation factor are reported below.

Sample type	Detection	Linearity equation	R^2 primary transition
Apple	Primary detection (223.1 – 126 m/z)	y = 1215.01x + 306.47	0.9995
	Confirmatory Detection (223.1 – 56 m/z)	y = 495.694x + 132.608	0.9995

Recovery and precision

Recovery and precision were tested by means of recovery tests at 2 spiking levels. Accuracy and precision result summaries are reported in the following table.

Primary detection (223.1 – 126 m/z)					
Sample type	**Fortification Level (mg/kg)**	**Recovery and precision per level**		**Overall recovery and precision**	
		Mean Recovery (%) n=5	**RSD (%) n=5**	**Mean Recovery (%) n=10**	**RSD (%) n=10**
Apple	0.01 (LOQ)	104.0	1.1	98.1	6.6
	0.2 (20xLOQ)	92.2	2.3		

Accuracy: the mean recovery per level found are in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 requirement (mean recovery per level in the range 70-110%).

Precision: the RSD% per level found are in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 (RSD% per level ≤ 20%).

Selectivity
The method was found to be selective for the determination of Acetamiprid on apple samples. No interfering signals higher than 30% of LOQ were detected in the control samples; this result is in compliance with the guideline requirements.

Matrix Effect
No matrix effect was assessed because the calibration curves were prepared using matrix matched analytical standards.

Limit of detection (LOD) and Limit of quantification (LOQ)
The Limit of Detection (LOD) is the lowest concentration at which the analyte produces an instrumental signal at least 3 times higher than the background noise.
The least concentrated standard injected that generate a signal at least 3 times higher than background noise was standard L1 (0.84 µg/L – 0.0034 mg/kg). It can be considered the Limit of detection.
This level corresponds to 30% of the target LOQ (0.01 mg/kg): at this level the signal to noise ratio was still higher than 3.

The Limit of Quantitation (LOQ) is defined the lowest concentration at which an acceptable recovery is obtained with an acceptable precision. The limit of quantification of the method was set to 0.01 mg/kg of Acetamiprid (corresponding to 2.62 µg/L in the final extract). Recovery and precision at this level resulted in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev.4 guidelines.

Analyte	**matrix**	**LOQ [mg/kg]**	**LOD [mg/kg]**
acetamiprid	Apple fruit	0.01	0.0034

Confirmation
A confirmation simultaneous to the primary detection using one additional HPLC-MS/MS transition for Acetamiprid was used. The additional transition monitored was 223.1 – 56 m/z.
Linearity, accuracy, precision and selectivity of the method were confirmed also using this additional transition.

Stability
All samples were analysed within 24 hours from preparation.

Conclusion

The methods were fully validated. Results of the validation of analytical methods was confirmed that this method is suitable for analysis the content of the test item ASA-01 in apple matrix.

A 2.1.1.3.2 HPLC-MS/MS (in oilseed rape whole plant and seeds)

A 2.1.1.3.2.1 Method validation

Comments of Evaluator:	The study was used in B7. The method validation is acceptable.

Reference:	KCP 5.1/13
Report	Acetamiprid – Residue Study on winter oilseed rape in Northern Europe – 2020, Report No. SDO-20-45215 (ANALYTICAL PHASE REPORT GLP-STUDY-20-26), Domingo S., 2023
Guideline(s):	SANCO 825/00 rev. 8.1 and SANCO/3029/99 rev. 4
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The objective of this Analytical Phase was to determine residue of acetamiprid on sample coming from the Field Phase of the Study SDO-20-45215 (Test Facility: Staphyt Spain S.L, Study Director: Susana Domingo). The analytical determinations were carried out using a HPLC-MS/MS method, validated according to SANCO 825/00 rev. 8.1 and SANCO/3029/99 rev. 4 guidelines.
The evaluated validation parameters were linearity, recovery and precision, limit of quantification and detection, selectivity, confirmation, matrix effect and stability.

Chromatographic conditions

- System operating: HPLC-MS/MS
- Instrument: Agilent HPLC 1290 Infinity II + Agilent spectrometer 6470A Triple Quad
- Column: Agilent Zorbax RRHD Clirse Plus C18/1.9 µm, 2.1x50 mm
- Column temperature: 40°C
- Flow: 0.600 mL/min
- Injection volume: 2.50 µL
- Mobile phase A: 100% LC-MS grade water + 0.1 % of Formic Acid
- Mobile phase B: 100% methanol UPLC grade + 0.1% of Formic Acid
- Elution:

Time (min)	%A	%B
0.50	95.0	5.00
2.50	50.0	50.0
3.00	0.0	100.0

- Divert valve: waste from 0 to 1.5 minutes, from 1.5 to 3 MS than waste until end of the run;
- Stop time: 6 minutes
- Source type: ESI
- Gas temperature: 350°C
- Gas flow (L/min): 11
- Nebulizer (psi): 60
- Sheath gas heater: 400°C
- Sheath gas flow (L/min): 12
- Capillary: 3000 V
- Vcharging: 0
- Acquiring mode: ESI positive, MRM (multi reaction monitoring.

Samples preparation
5 g aliquots of the homogenised sample were weighed in a 50 mL screw capped centrifuge PE test tube. In case of recovery test the sample was spiked at this stage. 20 mL of acetonitrile and 5 mL of water were added to the sample. The samples were thoroughly shaken for 2 minutes. 4 g of NaCl were added to the sample, the sample was further shaken for 1 minute and then centrifuged at 5000 RPM for 5 minutes. An aliquot of the final extract (1.5 mL) was transferred in a HPLC glass vial for the final determination.
All samples were analysed the same day of extraction.

Validation of analytical method

Linearity
Whole plant samples:
Acetamiprid linearity was checked in the range 0.5 – 251.5 µg/L (corresponding to 0.002 – 1.006 mg/kg on sample). 6 different concentration levels (single injection) were tested.
Seeds samples:
Acetamiprid linearity was checked in the range 0.5 – 50.30 µg/L (corresponding to 0.002 – 0.2012 mg/kg on sample). 5 different concentration levels (single injection) were tested.

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

Sample type	Detection	Linearity equation	R^2 primary transition
Whole Plant	Primary detection (223.1 – 126 m/z)	y = 201.6x + 39.97	0.9997
	Confirmatory Detection (223.1 – 56 m/z)	y = 100.9x + 20.18	0.9995
Seeds	Primary detection (223.1 – 126 m/z)	y = 155.6x – 0.6627	0.9997
	Confirmatory Detection (223.1 – 56 m/z)	y = 70.77x + 3.1353	0.9999

Recovery and precision
Recovery and precision were tested by means of recovery tests at 2 spiking levels. Accuracy and precision result summaries are reported in the following table.

Primary detection (223.1 – 126 m/z)					
Sample type	Fortification Level (mg/kg)	Recovery and precision per level		Overall recovery and precision	
		Mean Recovery (%) n=5	RSD (%) n=5	Mean Recovery (%) n=10	RSD (%) n=10
Whole Plant	0.01 (LOQ)	99.7	4.5	96.5	5.03
	0.7 (70xLOQ)	93.4	2.9		
Seeds	0.01 (LOQ)	96.9	4.3	94.1	4.9
	0.1 (10xLOQ)	91.3	3.6		

Accuracy: the mean recovery per level found are in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 requirement (mean recovery per level in the range 70-110%).

Precision: the RSD% per level found are in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4 (RSD% per level ≤ 20%).

Selectivity
The method was found to be selective for the determination of the analyte Acetamiprid in the selected matrix. No interfering signals higher than 30% of LOQ were detected in the control samples; this result is in compliance with the guideline requirements.

Matrix Effect
No matrix effect was assessed because the calibration curves were prepared using matrix matched analytical standards.

Limit of detection (LOD) and Limit of quantification (LOQ)
The Limit of Detection (LOD) is the lowest concentration at which the analyte produces an instrumental signal at least 3 times higher than the background noise.
The lowest concentration standard injected that generated a signal at least 3 times higher than background noise was standard L1 (0.5 µg/L – 0.00252 mg/kg). It can be considered the Limit of detection.
This level corresponds to 30% of the target LOQ (0.01 mg/kg (2.52 µg/L in the final extract): at this level the signal to noise ratio was still higher than 3.

The Limit of Quantitation (LOQ) is defined the lowest concentration at which an acceptable recovery is obtained with an acceptable precision. The limit of quantification of the method was set to 0.01 mg/kg of acetamiprid (corresponding to 2.52 µg/L in the final extract). Recovery and precision at this level resulted in compliance with SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev.4 guidelines.

Analyte	Matrix	LOQ [mg/kg]	LOD [mg/kg]
acetamiprid	Whole Plant	0.01	0.00252
	Seeds	0.01	0.00252

Confirmation
A confirmation simultaneous to the primary detection using one additional HPLC-MS/MS transition for Acetamiprid was used. The additional transition monitored was 223.1 – 56 m/z.
Linearity, accuracy, precision and selectivity of the method were confirmed also using this additional transition.

Stability
All the samples were analysed within 24 hours from preparation, therefore no experimental demonstration of extract stability was required.

Conclusion

The methods were fully validated. Results of the validation of analytical methods was confirmed that this method is suitable for analysis the content of the test item ASA-01 in rape matrix.

A 2.1.1.3.3 LC-MS/MS (in apple)

A 2.1.1.3.3.1 Method validation

Comments of Evaluator:	The study and the method validation has been accepted.

Reference: KCP 5.1/14

Report	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in apple, Report No. VAL/15/2023, Niewelt-Stasiak S., 2023
Guideline(s):	SANTE/2020/12830, Rev.2, 14 February 2023
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 its metabolite - acetamiprid-N-desmethyl (IM-2-1) in apple. Specimen extraction and determination of residues was performed using QuEChERS technique.
The analytical determinations were carried out using a LC-MS/MS method, validated according to SANTE/2020/12830, Rev.2, 14 February 2023 guideline. 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 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 0.001 µg/mL (because weigh of sample was equal 10 g).
The limit of quantification (LOQ) of the analytical method was 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.

LC-MS/MS settings
Instrument settings:
Liquid Chromatograph LCMS-8050 Shimadzu (WP-135/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.: B22352
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%
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

Initial sample preparation and homogenisation
Untreated samples for validation purposes were obtained from the other GLP study.
The field specimens arrived at the Test Facility in good conditions, frozen and were stored in a freezer at ≤ -18°C before analysis. All the samples were homogenized at Test Facility, using a knife grinder. The homogenized specimens were further stored at ≤ -18oC until beginning of analysis.

Extraction of acetamiprid and acetamiprid-n-desmethyl in apple
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 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 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.

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.

Validation of analytical method

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 1 to 500 ppb (0.0005-0.5 µg/mL) 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 di describes the vertical distance of measured values from the regression curve

according to:

$$di = yi - yyi$$

where:
yi - the measured value i
yyi - the estimated value which corresponds to yi 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 ≤30 %, and recovery at fortification level of 0.10 mg/kg (10xLOQ) should be in the range of 70 – 120% with RSD ≤ 20 %. RSD were determined only during validation process.

Acetamiprid

Transition: 223.10→126.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0048	96.0	0.05	0.045	91.0
	0.0047	93.7		0.048	95.8
	0.0049	98.5		0.047	94.7
	0.0048	96.1		0.049	98.3
	0.0049	97.7		0.050	99.9
Average	0.0048	96.4	Average	0.048	95.9
SD	0.000092	1.84	SD	0.0017	3.45
RSD [%]	1.91		**RSD [%]**	3.59	
Uncertanity [%]	8.1		**Uncertanity [%]**	10.8	

Transition: 223.10→ 56.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0049	98.0	0.05	0.046	92.4
	0.0047	94.6		0.049	97.6
	0.0046	92.5		0.048	96.8
	0.0046	92.2		0.049	98.8
	0.0049	97.2		0.050	100.1
Average	0.0047	94.9	Average	0.049	97.1
SD	0.00013	2.65	SD	0.0015	2.92
RSD [%]	2.79		**RSD [%]**	3.01	
Uncertanity [%]	11.6		**Uncertanity [%]**	8.3	

N-desmethyl-acetamiprid

Transition: 210.90→128.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0049	97.8	0.05	0.045	90.7
	0.0048	96.2		0.049	98.7
	0.0049	98.7		0.049	98.8
	0.0047	94.4		0.050	99.4
	0.0046	91.9		0.050	99.4
Average	0.0048	95.8	Average	0.049	97.4
SD	0.00014	2.71	SD	0.0019	3.78
RSD [%]	2.83		**RSD [%]**	3.88	
Uncertainty [%]	10.1		**Uncertainty [%]**	9.4	

Transition: 208.80→73.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0048	96.6	0.05	0.042	84.5
	0.0042	83.7		0.046	91.4
	0.0043	86.8		0.046	91.5
	0.0045	90.2		0.046	91.7
	0.0046	92.7		0.047	93.2
Average	0.0045	90.0	Average	0.045	90.5
SD	0.00025	5.03	SD	0.0017	3.41
RSD [%]	5.59		**RSD [%]**	3.77	
Uncertainty [%]	22.9		**Uncertainty [%]**	20.5	

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:

Matrix effects [%] = 100 * peak area or slope (matrix)/ peak area or slope (solvent) – 100

Matrix effects are considered significant if they exceed ±20%.

In case of this validation study, calibration standard at 100 ng/mL in solvent was additionally analysed and matrix effects were calculated according to the equation above.
Matrix effect [%] = -3.6
For acetamiprid and acetamiprid-N-desmethyl matrix effects calculated using equation are <±20%. Nevertheless, there was used matrix-matched calibration.

Limit of detection (LOD) and

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 0.001 µg/mL (because weigh of sample was equal 10 g).

Limit of quantification (LOQ)

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 0.001 mg/kg, for acetamiprid and N-desmethyl-acetamiprid in apple.
LOQ was successfully established at 0.005 mg/kg for acetamiprid and 0.005 mg/kg for N-desmethyl-acetamiprid (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.

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.

Stability of solutions
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 take 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.

Conclusion

The analytical method for determining the residues of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) in apple 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 0.005-0.05 mg/kg for acetamiprid-N-desmethyl (IM-2-1). Limit of detection was established at 0.001 mg/kg.

A 2.1.1.3.4 LC-MS/MS (in oilseed rape (seed, plant)

A 2.1.1.3.4.1 Method validation

Comments of Evaluator:	The study and the method validation has been accepted.

Reference:	KCP 5.1/15
Report	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in oilseed rape (seed, plant), Report No. VAL/14/2023, Niewelt-Stasiak S., 2023
Guideline(s):	SANTE/2020/12830, Rev.2, 14 February 2023
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 its metabolite - acetamiprid-N-desmethyl (IM-2-1) in oilseed rape (seed, plant). Specimen extraction and determination of residues was performed using QuEChERS technique.
The analytical determinations were carried out using a LC-MS/MS method, validated according to SANTE/2020/12830, Rev.2, 14 February 2023 guideline. 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 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 N-desmethyl-acetamiprid (IM-2-1), what corresponds to 0.0005 µg/mL (because weigh of sample was equal 5 g). Limit of quantification (LOQ) of the analytical method was 0.005 mg/kg for acetamiprid and 0.005 mg/kg for N-desmethyl-acetamiprid (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.

LC-MS/MS settings

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%
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

Initial sample preparation and homogenisation
Untreated samples for validation purposes were obtained from the other GLP study.
The field specimens arrived at the Test Facility in good conditions, frozen and were stored in a freezer at ≤ -18°C before analysis. All the samples were homogenized at Test Facility, using a knife grinder. The homogenized specimens were further stored at ≤ -18oC until beginning of analysis.

Extraction of acetamiprid and acetamiprid-n-desmethyl in oilseed rape (seed, plant)
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 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 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.

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.

Validation of analytical method

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 (0.0005-0.5 µg/mL) 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 di describes the vertical distance of measured values from the regression curve according to:

$$di = yi – yyi$$

where:
yi - the measured value i
yyi - the estimated value which corresponds to yi 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 0.005 mg/kg (LOQ) should be in the range of 60 – 120% with RSD ≤30 %, and recovery at fortification level of 0.05 mg/kg (10xLOQ) should be in the range of 70 – 120% with RSD ≤ 20 %. RSD were determined only during validation process.

Acetamiprid

Transition: 223.10→126.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0058	115.6	0.05	0.053	105.7
	0.0053	105.8		0.052	104.5
	0.0053	105.9		0.052	104.2
	0.0055	110.3		0.055	110.5
	0.0057	114.6		0.056	111.6
Average	0.0055	110.4	Average	0.054	107.3
SD	0.00023	4.66	SD	0.0018	3.51
RSD [%]	4.22		**RSD [%]**	3.27	
Uncertainty [%]	22.5		**Uncertainty [%]**	16.0	

Transition: 223.10→ 56.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0057	114.9	0.05	0.054	107.8
	0.0049	98.9		0.052	103.7
	0.0052	103.8		0.051	103.0
	0.0055	110.0		0.055	109.3
	0.0053	106.7		0.055	109.3
Average	0.0053	106.8	Average	0.053	106.6
SD	0.00030	6.06	SD	0.0015	3.06
RSD [%]	5.67		**RSD [%]**	2.87	
Uncertainty [%]	17.8		**Uncertainty [%]**	14.4	

N-desmethyl-acetamiprid

Transition: 210.90→128.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0054	108.6	0.05	0.053	105.5
	0.0051	101.8		0.055	109.1
	0.0047	93.3		0.052	104.3
	0.0056	111.1		0.056	112.3
	0.0051	102.1		0.058	116.9
Average	0.0052	103.4	Average	0.055	109.6
SD	0.00035	6.94	SD	0.0026	5.16
RSD [%]	6.72		**RSD [%]**	4.71	
Uncertainty [%]	15.0		**Uncertainty [%]**	21.4	

Transition: 208.80→73.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0055	109.0	0.05	0.053	106.2
	0.0045	90.0		0.052	103.7
	0.0045	90.9		0.050	99.8
	0.0049	98.2		0.055	110.6
	0.0048	96.8		0.053	106.5
Average	0.0048	97.0	Average	0.053	105.4
SD	0.00038	7.61	SD	0.0020	3.98
RSD [%]	7.85		**RSD [%]**	3.78	
Uncertainty [%]	16.8		**Uncertainty [%]**	13.2	

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:

Matrix effects [%] = 100 * peak area or slope (matrix)/ peak area or slope (solvent) – 100

Matrix effects are considered significant if they exceed ±20%.

In case of this validation study, calibration standard at 100 ng/mL in solvent was additionally analysed and matrix effects were calculated according to the equation above.
Matrix effect [%] = -70.3
For acetamiprid and acetamiprid-N-desmethyl matrix in oilseed rape seed and plant matrix effect calculated using equation exceed ±20%. To compensate matrix effect, there was used matrix-matched calibrations.

Limit of detection (LOD) and
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 N-desmethyl-acetamiprid (IM-2-1), what corresponds to 0.0005 µg/mL (because weigh of sample was equal 5 g).

Limit of quantification (LOQ)
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 0.001 mg/kg, for acetamiprid and N-desmethyl-acetamiprid in oilseed rape (seed, plant).
LOQ was successfully established at 0.005 mg/kg for acetamiprid and 0.005 mg/kg for N-desmethyl-acetamiprid (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.

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.

Stability of solutions
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 take 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.

Conclusion

The analytical method for determining the residues of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) in oilseed rape (seed, plant) 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 for acetamiprid and 0.005-0.05 mg/kg for N-desmethyl-acetamiprid (IM-2-1). Limit of detection was established at 0.001 mg/kg.

A 2.1.1.3.5 LC-MS/MS (in honey)

A 2.1.1.3.5.1 Method validation

Comments of Evaluator:	The study and the method validation has been accepted.

Reference:	KCP 5.1./16
Report	Validation of an analytical method for the determination of residues of acetamiprid and acetamiprid-N-desmethyl in honey, Report No. VAL/13/2023,

	Niewelt-Stasiak S., 2023
Guideline(s):	SANTE/2020/12830, Rev.2, 14 February 2023
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 its metabolite - acetamiprid-N-desmethyl (IM-2-1) in honey. Specimen extraction and determination of residues was performed using QuEChERS technique.
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 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 N-desmethyl-acetamiprid (IM-2-1), what corresponds to 0.0005 µg/mL (because weigh of sample was equal 5 g). Limit of quantification (LOQ) of the analytical method was 0.005 mg/kg for acetamiprid and 0.005 mg/kg for N-desmethyl-acetamiprid (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.

LC-MS/MS settings
Instrument settings:
Liquid Chromatograph LCMS-8050 Shimadzu (WP-135/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%
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

Initial sample preparation and homogenisation
Untreated samples for validation purposes were obtained from commercial source.
The field specimens arrived at the Test Facility in good conditions and were further stored at ≤ -18°C until

beginning of analysis.

Extraction of acetamiprid and acetamiprid-n-desmethyl in apple
5 g of the homogenized sample was weighed into a 50 mL centrifuge tube. 10 mL of acetonitrile and 10 mL of 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 dehydrate, 0.5 g disodium hydrogencitrate sesquihydrate), the mixture was shaken 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 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.

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 analyzed 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.

Validation of analytical method

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 di describes the vertical distance of measured values from the regression curve according to:

$$di = yi – yyi$$

where:
yi - the measured value i
yyi - the estimated value which corresponds to yi 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 0.005 mg/kg (LOQ) should be in the range of 60 – 120% with RSD ≤30 %, and recovery at fortification level of 0.05 mg/kg (10xLOQ) should be in the range of 70 – 120% with RSD ≤ 20 %. RSD were determined only during validation process.

Acetamiprid

Transition: 223.10→126.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0050	99.6	0.05	0.045	90.5
	0.0049	97.3		0.043	86.8
	0.0046	92.4		0.045	90.6
	0.0044	88.5		0.044	87.3
	0.0045	89.2		0.043	86.0
Average	0.0047	93.4	Average	0.044	88.2
SD	0.00024	4.89	SD	0.0011	2.15
RSD [%]	5.24		**RSD [%]**	2.44	
Uncertainty [%]	16.8		**Uncertainty [%]**	24.1	

Transition: 223.10→ 56.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0051	102.4	0.05	0.048	96.0
	0.0049	97.3		0.047	93.8
	0.0048	95.5		0.047	93.9
	0.0047	93.4		0.046	91.3
	0.0048	96.3		0.047	93.2
Average	0.0048	97.0	Average	0.047	93.6
SD	0.00017	3.36	SD	0.00084	1.67
RSD [%]	3.46		**RSD [%]**	1.79	
Uncertainty [%]	9.2		**Uncertainty [%]**	13.2	

N-desmethyl-acetamiprid

Transition: 210.90→128.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0048	97.0	0.05	0.045	89.1
	0.0043	86.7		0.044	88.6
	0.0044	88.5		0.044	88.4
	0.0041	81.4		0.043	85.2
	0.0044	87.2		0.044	87.2
Average	0.0044	88.2	Average	0.044	87.7
SD	0.00028	5.63	SD	0.00078	1.57
RSD [%]	6.39		**RSD [%]**	1.78	
Uncertainty [%]	26.9		**Uncertainty [%]**	24.9	

Transition: 208.80→73.10

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.005	0.0043	86.5	0.05	0.044	88.2
	0.0045	90.7		0.043	86.5
	0.0043	86.7		0.044	87.1
	0.0043	86.9		0.043	85.9
	0.0043	85.9		0.043	86.7
Average	0.0044	87.3	Average	0.043	86.9
SD	0.00010	1.91	SD	0.00043	0.86
RSD [%]	2.19		**RSD [%]**	0.99	
Uncertainty [%]	25.7		**Uncertainty [%]**	26.3	

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:

Matrix effects [%] = 100 * peak area or slope (matrix)/ peak area or slope (solvent) – 100

Matrix effects are considered significant if they exceed ±20%.

In case of this validation study, calibration standard at 100 ng/mL in solvent was additionally analysed and matrix effects were calculated according to the equation above.
Matrix effect [%] = 6.7
For acetamiprid and acetamiprid-N-desmethyl matrix effects calculated using equation are < 20%. Nevertheless, there was used matrix-matched calibration.

Limit of detection (LOD) and

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 N-desmethyl-acetamiprid (IM-2-1), what corresponds to 0.0005 µg/mL (because weigh of sample was equal 5 g).

Limit of quantification (LOQ)

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.
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.

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.

Stability of solutions
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 take 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.

Conclusion

The analytical method for determining the residues of acetamiprid and N-desmethyl-acetamiprid (IM-2-1) in honey 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 (µg/g) for acetamiprid and 0.005 - 0.05 mg/kg for N-desmethyl-acetamiprid (IM-2-1). Limit of detection was established at 0.001 mg/kg.

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

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

No new or additional studies have been submitted.

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

Please refer to point A 2.1.1.3.5.

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

No new or additional studies have been submitted.

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

A 2.1.2.4.1.1 Independent laboratory validation

Comments of Evaluator:	The ILV has been accepted in the context of other previous PL assessment (unprotected study from DML Los Ovados 200 SE acc. to the applicant information)

Reference:	KCP 5.2
Report	Acetamiprid: Independent Laboratory Validation of an Analytical Method for the Determination in Drinking Water, Dr. Matthias Eichler, 2018, 133112101
Guideline(s):	Yes (SANCO/825/00 rev. 8.1)
Deviations:	Deviation to the Study Plan: Pure water was used for pre-conditioning and

	rinsing of SPE columns and for reconstituting samples after evaporation. Reason for the Deviation: Human error Presumable Effect on the Study: Pure water was taken from a water purification system. This type of water is even purer than deionised water and therefore considered to have no negative effect on the outcome of this study
GLP:	Yes
Acceptability:	Yes

Materials and methods

The purpose of this study was to independently validate the analytical method "Validation of the Methods of Analysis used for the Determination of Acetamiprid in Water, in Compliance with Good Laboratory Practice, and referencing SANCO/3029/99."(Norris, D.; 2017; Study No. DNA4037) to determine Acetamiprid in drinking water. The analyte was extracted from drinking water using a Mega Bond Elut C18 and a Sep-Pak plus C18 solid phase extraction (SPE) cartridge. The eluate was evaporated to dryness under reduced pressure and reconstituted in solvent. The analyte concentration in the final solutions was determined via LC-MS/MS technique.

Results and discussions

The relative standard deviation of recoveries at each fortification level should be less or equal than 20% and the mean recoveries at each fortification level should be within the range of 70 - 120% as demanded in guideline SANCO/825/00 rev.8.1. The limit of quantification (LOQ) is determined as the lowest ana-lyte concentration at which acceptable recovery (70 to 110 % of nominal) with a coefficient of variation ≤ 20 % has been obtained. Specificity was established by monitoring two different mass fragments, one as a quantifier (223 → 126 m/z) and one as a qualifier (223 → 90 m/z). In all matrices tested, the mean recovery values were between 70% and 110%. The relative standard deviations (RSD) for all fortification levels were below 20%. The detailed results are given in the table below.

Table A 17: Recovery results from independent laboratory validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 6)	Mean Recovery (%)	RSD (%)	Comment
Water	Acetamiprid	10 µg/L	89	1	quantifier (223 → 126 m/z)
		1.0 µg/L	91	8	
		0.1 µg/L	84	6	
		0.05 µg/L	82	3	
	Acetamiprid	10 µg/L	91	1	qualifier (223 → 90 m/z)
		1.0 µg/L	92	8	
		0.1 µg/L	80	5	
		0.05 µg/L	73	3	

Table A 18: Characteristics for the analytical method used for independent laboratory validation of active substance residues in matrix

	Acetamiprid
Specificity	The method must be capable of determining the active ingredient in the presence of sample matrix. Two mass transitions were validated using liquid chromatography with MS detection. One mass transition was used for quantification and one was used for confirmation. Data for

	another mass transition was recorded but was not evaluated. Specificity was established by monitoring two different mass fragments, one as a quantifier (223 → 126 m/z) and one as a qualifier (223 → 90 m/z). Interference: There was no interference from blank values and therefore the recommendation by SANCO guideline (< 30 % of the mean peak area at LOQ level) is fulfilled.
Calibration (type, number of data points)	The lower margin of the linearity test should be less or equal to 30% of the LOQ and the upper margin should be higher by at least 20% of the 10-fold LOQ concentrations or the highest test concentration in the final extracts. Linearity was assessed by investigating the correlation between peak area or height of the standard solutions to their corresponding concentration. At least five concentrations were measured. Linear calibration is preferred and where a non-linear calibration is used, a justification must be provided.
Calibration range	0.7 to 100 µg/L
Assessment of matrix effects is presented	The peak areas obtained from measurement of the solvent standards and matrix-matched standards were compared. The mean difference between peak areas was 7 % for quantifier and qualifier mass transition. Therefore, it was decided to use calibration standards prepared from
Limit of determination/quantification	0.05 µg/L (LOQ)

Conclusion

For the method validation purpose, drinking water was spiked at four concentration levels. The validity criteria linearity, accuracy, precision and repeatability were fulfilled for analysis of the test item in drinking water.

Comments of Evaluator:	The ILV has been accepted in the context of other previous PL assessment (un-protected study from DML Los Ovados 200 SE acc. to the applicant information)

Reference:	KCP 5.2
Report	IM-1-5 (Metabolite of Acetamiprid): Independent Laboratory Validation of an Analytical Method for the Determination in Drinking Water, Dr. Matthias Eichler, 2018, 133141101
Guideline(s):	Yes (SANCO/825/00 rev. 8.1)
Deviations:	Deviation to the Study Plan: Acquity UPLC BEH C18 (100*2.1 mm; 1.7 µm) Reason for Deviation: Error, the Acquity column was another column used in the pre-experiments. Presumable Effect on the Study: None, both columns showed similar performances in pre-tests. The method was fully validated using the Acquity column.
GLP:	Yes
Acceptability:	Yes

Materials and methods

An analytical method to determine IM-1-5 (Metabolite of Acetamiprid) in drinking water was provided by the Sponsor ("Validation of the Methods of Analysis used for the Determination of a Metabolite of Acetamiprid in Drinking Water, in Compliance with Good Laboratory Practice, and referencing SANCO/825/00 rev. 8.1."(Norris, D.; 2018; Study No. DNA4518)). The analytical method was independently

validated at the performing laboratory. Any addition or modification of the original method was discussed with the Sponsor´s monitor before implementation and is reported and justified in this report. The method was validated for drinking water with a Limit of Quantification (LOQ) of 0.05 µg/L.

Results and discussions

The relative standard deviation of recoveries at each fortification level should be less or equal than 20% and the mean recoveries at each fortification level should be within the range of 70 - 120% as demanded in guideline SANCO/825/00 rev.8.1. The limit of quantification (LOQ) is determined as the lowest ana-lyte concentration at which acceptable recovery (70 to 110 % of nominal) with a coefficient of variation ≤ 20 % has been obtained. Specificity was established by monitoring two different mass fragments, one as a quantifier (223 → 126 m/z) and one as a qualifier (223 → 90 m/z). In all matrices tested, the mean recovery values were between 70% and 110%. The relative standard deviations (RSD) for all fortification levels were below 20%. The detailed results are given in the table below.

Table A 19: Recovery results from independent laboratory validation of analyte using the analytical method

Matrix	Analyte	Fortification level (mg/kg) (n = 6)	Mean Recovery (%)	RSD (%)	Comment
Water	IM-1-5	0.5 µg/L	105	2	quantifier (198 → 126 m/z)
		0.05 µg/L	98	86	
	IM-1-5	0.5 µg/L	103	11	qualifier (198 → 90 m/z)
		0.05 µg/L	99	86	

Table A 20: Characteristics for the analytical method used for independent laboratory validation of active substance residues in matrix

	IM-1-5
Specificity	The method must be capable of determining the active ingredient in the presence of sample matrix. Two mass transitions were validated using liquid chromatography with MS detection. One mass transition was used for quantification, the other was used for confirmation. Linearity: The lower margin of the linearity test was less or equal to 30% of the LOQ and the upper margin was higher by at least 20% of the 10 fold LOQ concentrations in the final extracts. Linearity was assessed by investigating the correlation between peak area of the standard solutions to their corresponding concentration. Nine concentrations were measured. Linear calibration was used. Specificity was established by monitoring two different mass fragments, one as a quantifier (198 → 126 m/z) and one as a qualifier (198 → 90 m/z). The interference from blank values was < 30 % of the LOQ
Calibration (type, number of data points)	The control samples and samples prepared at LOQ level were evaluated using calibration data of the lower concentration range; i.e. 0.015 to 0.25 µg/L. The fortified samples of the 10*LOQ level were evaluated using calibration data of the higher concentration range; i.e. 0.015 to 5 µg/L.
Calibration range	0.015 to 5 µg/L (high range) 0.015 to 0.25 µg/L (low range)
Assessment of matrix effects is presented	The peak areas obtained from measurement of the solvent standards and matrix-matched standards were compared. The mean difference between peak areas was 30%. Therefore, it was decided to use the matrix-matched calibration standards for calibration and evaluation.
Limit of determination/quantification	0.05 µg/L (LOQ)

Conclusion

For the method validation purpose, drinking water was spiked at two concentration levels. The validity criteria linearity, accuracy, precision and repeatability were fulfilled for analysis of the test item in drinking water.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.