

FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: AKO 600 SC

Product name(s): Tanger 600 SC, Libretto 600 SC

Chemical active substance:

Aclonifen: 600 g/L

Central Zone

Zonal Rapporteur Member State: Poland

Co-Rapporteur Member State: Hungary, Slovakia, Germany,
Romania

CORE ASSESSMENT

(authorization)

Applicant: Innvigo Sp. z o.o.

Submission date: 18.11.2024

zRMS Assessment : 28/06/2025

Following commenting period: 01/10/2025

Verification of reference list: 28/10/2025

Final Version:29/06/2026

Version history

When	What
June 2025	zRMS evaluation
October 2025	Following commenting period
October 2025	Verification of reference list
June 2026	Final version

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5 Analytical methods

According to Regulation 1107/2009 data protection for data at European level lasts for 10 years. Due to the fact that the aclonifen annex I was approved on 1/08/2009, data protection has expired 1/08/2019. Therefore, Innvigo can use the European data for aclonifen for the registration of the product AKO 600 SC [Libretto 600 SC, Tanger 600 SC]

5.1 Conclusion and summary of assessment

State whether submitted data are sufficient for evaluation. Data gaps and conditions for authorization should be listed, if appropriate.

Sufficiently sensitive and selective analytical methods are available for the active substance(s) and relevant impurities 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.

- Noticed **data gap**:

According to Commission Regulation (EU) No Reg. No 283/2013 and Reg. No 284/2013 and SAN-TE/2020/12830 rev.1, a fully ILV validated analytical method for monitoring aclonifen residues in kidney, liver is required. **Data gap**. The sponsor has declared that the ILV study SENCIUC, 2025 S24-105061 is ongoing and will be available in June/July 2025. It may be evaluated in post-registration at national level.

Commodity/crop	Supported/ Not supported
Potato	supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

Comments of zRMS:	The analytical method code: ICB/106/2023 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANCO/3030/99 rev.5. The results of analytical method validation confirm that this method is suitable for determination of aclonifen and phenol as impurity. The method is successfully validated and accepted.
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Reference: KCP 5.1.1

Report Validation of analytical method for CHR/H/AKO 600 SC for determination of aclonifen and phenol as impurity; Study code: ICB/106/2023; Górka Iwona, MSc ICB Pharma

Guideline(s):	- Revision of 30 April 2013 draft, 4 November 2013; Guidance document for single laboratory validation of quantitative analytical methods used in support of pre- and post-registration data requirements for plant protection and biocidal products, - The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture July 2009; Quality Control of Pesticide Products, - SANCO/3030/99 rev.5 22/03/19 Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013.
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

Materials used for:

- Calibration: standard stock solution of aclonifen in acetonitrile (weight - 10.0 mg) and 5 working standard solutions of aclonifen in acetonitrile with increasing standard solution volumes
- Determination recovery: standard stock solution of aclonifen in acetonitrile (weight – 49.0 mg)

Validation was carried out according to Standard Operational Procedure SPB/179. Content of aclonifen at a level of 600 g/L in the test item was determined accordingly by liquid chromatography with diode array detection (HPLC-DAD). In this chromatography 2 chromatographic systems were used: primary and secondary.

For validation method for determination of aclonifen were prepared two series of measurements of the test item, one without addition standard and second with addition standard. 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. To determine recovery, was prepared series of measurements (n=5) of the test item with the addition of standard. Calibration curve of active ingredient was prepared to determine the linear range. The average recovery and precision for validation level LOQ, was determined by prepared series of measurements (n=5) of solvent (acetonitrile) with standard addition. The average recovery and precision for validation level ULOQ, was determined by prepared series of measurements (n=5) of solvent (acetonitrile) with standard addition. Afterwards the sample was 4-fold diluted to fit the linear range of active ingredient. Specificity of the method was evaluated based on the analysis of sample chromatogram against chromatogram of standard aclonifen and peak purity.

Validation parameters were determined in according to the requirements in Revision of 30 April 2013 draft, 4 November 2013; Guidance document for single laboratory validation of quantitative analytical methods used in support of pre- and post-registration data requirements for plant protection and biocidal products, The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture July 2009; Quality Control of Pesticide Products, SANCO/3030/99 rev.5 22/03/19 Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013.

Equipment and materials.

- acetonitrile (Merck),
- 85% phosphoric acid (VWR),
- aclonifen standard; Sigma Aldrich; batch BCCH7675, prod. date: June 15, 2022; exp.

date: May, 2027; storage conditions: $20 \pm 4^{\circ}\text{C}$,

- standard stock solution of aconifen in acetonitrile (for calibration) (Table 3),
- working standard solutions of aconifen in acetonitrile (for calibration) (Table 4),
- standard stock solution of aconifen in acetonitrile (for recovery) (Table 5),
- analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16,
- ultrasonic bath, W/29 (Branson, Taiwan),
- liquid chromatograph with diode array detection (Shimadzu, Japan), WP/19, WP/42,
- chromatography column type C18, 250 mm x 4,6 mm, 5 μm ; (Zorbax Eclipse Plus, Agilent), K/11/HPLC,
- chromatography column type C18, 100Å, 150 mm x 4,6 mm, 5 μm ; (Luna Omega Polar, Phenomenex), K/15/HPLC,
- chromatographic vials 1.5 mL with septa buthyl/Teflon,
- volumetric flasks A class 10 mL,
- measuring syringes 10 μL , 100 μL , 250 μL , 500 μL , 1000 μL .

Chromatography parameters.

Chromatography parameters were determined during preliminary assessment.

4.1.3.1. Primary chromatographic system.

Column: K/11/HPLC

Analysis time: 10 min

Flow: 1.4 mL/min

Column temperature: 25°C

Injection: 20 μL

Detector DAD: 237 nm

Mobile phase: A – Acetonitrile, D – 0.1% H_3PO_4

Isocratic program: phase A - 80%, D – 20%

Secondary chromatographic system.

Column: K/15/HPLC

Analysis time: 10 min

Flow: 1.4 mL/min

Column temperature: 25°C

Injection: 20 μL

Detector DAD: 237 nm

Mobile phase: A – Acetonitrile, D – 0.1% H_3PO_4

Isocratic program: phase A - 70%, phase D – 30%

Linearity.

In order to check the linearity of aconifen, calibration curve was prepared using standard solutions with concentrations contained in Table 4. A graph of the peak area to the concentration of aconifen was plotted. The resulting curve is linear in the tested concentrations. Linearity range of aconifen is from 1.076 to 312.96 $\mu\text{g/mL}$. Correlation coefficient R^2 is 0.9999991 (Figure 3) and the linear regression is described by equation: $f(x) = 1.83502 \cdot 10^{-5}x - 0.0435281$ for primary chromatographic system. Correlation coefficient R^2 is 0.9999926 (Figure 9) and the linear regression is described by equation: $f(x) = 1.85925 \cdot 10^{-5}x - 0.152390$ for secondary chromatographic system.

Specificity.

Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard aconifen 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.

Sample preparation for determination average content of aconifen and precision.

Sample of test item was standardized by gentle mixing for 1 minute. Approximately 50 mg of test item was weighed out into 10 mL volumetric flask and was filled up to the mark with acetonitrile. Table 10 contains masses of weights of the test item used for determination average content of active ingredient and precision. The contents of the flask was sonicated for 5 minutes. Afterwards 200 μL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with acetonitrile. Then final solu-

tion was placed in chromatographic vial and analysed according to conditions given in chapter 4.1.3. The test item was prepared in five repetitions.

Sample preparation for determination recovery for aclonifen.

Sample of test item was standardized by gentle mixing for 1 minute. Approximately 50 mg of test item was weighed out into 10 mL volumetric flask. Then 1200 µL 4.89 mg/mL standard stock solution of aclonifen was added and volumetric flask was filled up to the mark with acetonitrile. Table 13 contains masses of weights of the test item used for determination recovery. The contents of the flask was sonicated for 5 minutes. Afterwards 200 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with acetonitrile. Then final solution was placed in chromatographic vial and analysed according to conditions given in chapter 4.1.3. The test item was prepared in five repetitions.

Solvent (acetonitrile) preparation for determination of average recovery and precision for validation level LOQ for aclonifen.

110 µL 4.89 mg/mL standard stock solution of aclonifen was added to 10 mL volumetric flask and filled up to the mark with acetonitrile. The contents of the flask was sonicated for 5 minutes. Afterwards 200 µL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with acetonitrile. Then final solution was placed in chromatographic vial and analysed according to conditions given in chapter 4.1.3. Solvent was prepared in five repetitions.

Solvent (acetonitrile) preparation for determination average recovery and precision for validation level ULOQ for aclonifen.

640 µL 4.89 mg/mL standard stock solution of aclonifen was added to 10 mL volumetric flask and filled up to the mark with acetonitrile. The contents of the flask was sonicated for 5 minutes. Afterwards 2.5 mL of solution was transferred into 10 mL volumetric flask and was filled up to the mark with acetonitrile. The sample was 4-fold diluted to fit the linear range of active ingredient. Then final solution was placed in chromatographic vial and analysed according to conditions given in chapter 4.1.3. Solvent was prepared in five repetitions

Comments of zRMS:

SUMMARY					
Table 1. Parameters of the analytical method for determination of aclonifen.					
Results for primary chromatographic system.					
Active ingredient			Linearity		
aclonifen			R ² = 0.9999991		
Validation level	Active ingredient	Precision [%]	Horwitz ratio	Recovery [%]	Standard addition [%]
100% without standard addition	aclonifen	0.73	0.49	-	-
100% with standard addition (20-30%)	aclonifen	-	-	97.73-102.13	23.53
LOQ	aclonifen	0.49	0.18	101.1 (average)	-
ULOQ	aclonifen	0.58	0.42	100.0 (average)	-
Results for secondary chromatographic system.					
Active ingredient			Linearity		
aclonifen			R ² = 0.9999926		
Validation level	Active ingredient	Precision [%]	Horwitz ratio	Recovery [%]	Standard addition [%]
100% without standard addition	aclonifen	0.39	0.26	-	-
100% with standard addition (20-30%)	aclonifen	-	-	97.76-102.03	23.45
LOQ	aclonifen	0.97	0.37	92.0 (average)	-
ULOQ	aclonifen	0.51	0.37	100.2 (average)	-

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances Aclonifen in plant protection product AKO 600 SC

	Aclonifen
Author(s), year	Górka I. 2023
Principle of method	HPLC-DAD
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	Primary chromatographic system: $R^2 = 0.9999991$
	Secondary chromatographic system: $R^2 = 0.9999926$
Precision – Repeatability Mean (100% w/o) n = 5 (%RSD)	Primary chromatographic system: 0.73%
	Secondary chromatographic system: 0.39%
Precision – Repeatability Mean (LOQ) n = 5 (%RSD)	Primary chromatographic system: 0.49%
	Secondary chromatographic system: 0.97%
Precision – Repeatability Mean (ULOQ) n = 5 (%RSD)	Primary chromatographic system: 0.58%
	Secondary chromatographic system: 0.51%
Accuracy (100%) n = 5 (% Recovery)	Primary chromatographic system: 97.73 - 102.13%
	Secondary chromatographic system: 97.76 - 102.03%
Accuracy (LOQ) n = 5 (% Recovery)	Primary chromatographic system: 101.1 (average)%
	Secondary chromatographic system: 92.0 (average)%
Accuracy (ULOQ) n = 5 (% Recovery)	Primary chromatographic system: 100.0 (average)%
	Secondary chromatographic system: 100.2 (average)%
Interference/ Specificity	Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard aclonifen 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.
Comment	-

Conclusion

Aclonifen's linearity, precision and accuracy parameters are in the acceptance range and complies with EU requirements. Results were compared to values described in Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013 - SANCO/3030/99 rev.5 22 March 2019,

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

Comments of zRMS:	The analytical method code: ICB/106/2023 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANCO/3030/99 rev.5. The results of analytical method validation confirm that this method is suitable for determination of aclonifen and phenol as impurity. The method is successfully validated and accepted.
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Reference: KCP 5.1.1

Report Validation of analytical method for CHR/H/AKO 600 SC for determination of aclonifen and phenol as impurity; Study code: ICB/106/2023; Górka Iwona, MSc ICB Pharma

Guideline(s):
- Revision of 30 April 2013 draft, 4 November 2013; Guidance document for single laboratory validation of quantitative analytical methods used in support of pre- and post-registration data requirements for plant protection and biocidal products,
- The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture July 2009; Quality Control of Pesticide Products,
- SANCO/3030/99 rev.5 22/03/19 Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013.

Deviations: No

GLP: Yes

Acceptability: Yes

Materials and methods

Materials used for:

- Calibration: standard solution of phenol in acetonitrile (weight - 10.2 mg) and 5 working standard solutions of phenol in acetonitrile with increasing standard solution volumes
- Determination recovery: working standard solution of phenol in acetonitrile (concentration – 1.02 mg/mL)

Validation was carried out according to Standard Operational Procedure SPB/179. Content of phenol in the test item was determined by liquid chromatography with diode array detection (HPLC-DAD). In this chromatography 2 chromatographic systems were used: primary and secondary.

For validation method for determination of phenol in the formulation SC which contains aclonifen as active ingredient were prepared two series of measurements of the test item, one without addition standard and second with addition standard. To determine the average content of phenol (validation level 100%) and precision, was prepared series of measurements (n=5) of the test item without standard addition. To determine recovery, was prepared series of measurements (n=5) of the test item with the addition of standard. Calibration curve of phenol was prepared to determine the linear range. The average recovery and precision for validation level LOQ, was determined by prepared series of measurements (n=5) of solvent (acetonitrile) with standard addition. Specificity of the method was evaluated based on the analysis of sample chromatogram against chromatogram of standard phenol and peak purity.

Validation parameters were determined in according to the requirements in Revision of 30 April 2013

draft, 4 November 2013; Guidance document for single laboratory validation of quantitative analytical methods used in support of pre- and post-registration data requirements for plant protection and biocidal products, The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture July 2009; Quality Control of Pesticide Products, SANCO/3030/99 rev.5 22/03/19 Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013.

Equipment and materials.

- acetonitrile (Merck),
- 85% phosphoric acid (VWR),
- phenol standard; batch BCCG0109, prod. date: August 17, 2021; exp. date: April, 2026; storage conditions: 4±2°C,
- phenol standard stock solution in acetonitrile (Table 6),
- working standard solution of phenol in acetonitrile (for calibration) (Table 7),
- working standard solutions of phenol in acetonitrile (for calibration) (Table 8),
- working standard solution of phenol in acetonitrile (for recovery) (Table 9),
- analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16,
- ultrasonic bath, W/29 (Branson, Taiwan),
- syringe filters with a pore diameter of 0.45 µm,
- liquid chromatographs with diode array detection (Shimadzu, Japan), WP/19, WP/42,
- chromatography column type C18, 100Å, 250 mm x 4,6 mm, 5 µm (Luna, Phenomenex), K/17/HPLC,
- chromatography column type C18, 100Å, 250 mm x 4,6 mm, 5 µm; (Luna, Phenomenex), K/1/HPLC,
- chromatographic vials 1,5 mL with septa buthyl/teflon,
- volumetric flasks class A, 10 mL,
- measuring syringes 10 µL, 50 µL, 500 µl and 1000 µL.

Primary chromatographic system.

Column: K/17/HPLC
Analysis time: 45 min
Flow: 1.4 mL/min
Column temperature: 25 °C
Injection: 30 µL
Detector DAD: 270 nm
Mobile phase: A – Acetonitrile, D – 0.1% H₃PO₄
Gradient program: 10 min phase A - 25%, phase D – 75%
15 min phase A - 100%, phase D – 0%
20 min phase A - 100%, phase D – 0%
25 min phase A - 100%, phase D – 0%
35 min phase A - 25%, phase D – 75%
45 min phase A - 25%, phase D – 75%

Secondary chromatographic system.

Column: K/1/HPLC
Analysis time: 45 min
Flow: 1.2 mL/min
Column temperature: 25 °C
Injection: 30 µL
Detector DAD: 270 nm
Mobile phase: A – Acetonitrile, D – 0.1% H₃PO₄
Gradient program: 7 min phase A - 25%, phase D – 75%
15 min phase A - 100%, phase D – 0%
20 min phase A - 100%, phase D – 0%
25 min phase A - 100%, phase D – 0%

35 min phase A - 25%, phase D – 75%

45 min phase A - 25%, phase D – 75%

Linearity.

In order to check the linearity of phenol, calibration curve was prepared using standard solutions with concentrations contained in Table 8. A graph of the peak area to the concentration of phenol was plotted. The resulting curve is linear in the tested concentrations. Linearity range of phenol is from 0.184 to 20.400 µg/mL. Correlation coefficient R² is 0.9999731 (Figure 59) and the linear regression is described by equation: $f(x) = 5.36705 \cdot 10^{-5}x - 0.0202411$ for primary chromatographic system. Correlation coefficient R² is 0.9999527 (Figure 65) and the linear regression is described by equation: $f(x) = 4.44378 \cdot 10^{-5}x - 0.0188189$ for secondary chromatographic system.

Specificity.

Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard phenol and peak purity (Figures 71-74). Analysis showed no overlapping of determined impurity signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.

Sample of the test item preparation for determination average content of phenol.

Sample of test item was standardized by gentle mixing for 1 minute. Approximately 300 mg of test item was weighed out into 10 mL volumetric flask and was filled up to the mark with acetonitrile. Table 20 contains masses of weights of the test item used for determination average content of phenol. The contents of the flask was sonicated for 5 minutes. Afterwards the suspension was filtered through a 0.45 µm filter. Then final solution was placed in chromatographic vial and analysed according to the conditions given in chapter 4.2.3. The test item was prepared in five repetitions.

Sample preparation for determination recovery for phenol.

Sample of test item was standardized by gentle mixing for 1 minute. Approximately 300 mg of test item was weighed out into 10 mL volumetric flask. Then 80 µL 10.2 µg/mL standard working solution of phenol was added and volumetric flask was filled up to the mark with acetonitrile. Table 23 contains masses of weights of the test item used for determination recovery. The content of the flask was sonicated for 5 minutes. Afterwards the suspension was filtered through a 0.45 µm filter. Then final solution was placed in chromatographic vial and analysed according to conditions given in chapter 4.2.3. The test item was prepared in five repetitions.

Solvent (acetonitrile) preparation for determination of average recovery and precision for validation level LOQ for phenol.

18 µL 102.0 µg/mL standard working solution of phenol was added to 10 mL volumetric flask and filled up to the mark with acetonitrile. The content of the flask was sonicated for 5 minutes. Afterwards the suspension was filtered through a 0.45 µm filter. Then final solution was placed in chromatographic vial and analysed according to conditions given in chapter 4.2.3. Solvent was prepared in five repetitions.

Comments of zRMS:

Table 2. Parameters of the analytical method for determination of phenol as impurity.					
Results for primary chromatographic system.					
Impurity			Linearity		
phenol			R ² = 0.9999731		
Validation level	Impurity	Precision [%]	Horwitz ratio	Recovery [%]	Standard addition [%]
100% without standard addition	phenol	3.92	0.52	-	-
100% with standard addition (20-30%)	phenol	-	-	76.63-123.70	25.53
LOQ	phenol	7.35	0.90	103.8 (average)	-
Results for secondary chromatographic system.					
Impurity			Linearity		
phenol			R ² = 0.9999527		
Validation level	Impurity	Precision [%]	Horwitz ratio	Recovery [%]	Standard addition [%]
100% without standard addition	phenol	1.83	0.24	-	-
100% with standard addition (20-30%)	phenol	-	-	85.26-106.05	24.94
LOQ	phenol	6.09	0.75	103.5 (average)	-

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of the relevant impurities in plant protection product (PPP) AKO 600 SC

	Phenol
Author(s), year	Górka Iwona, 2023
Principle of method	HPLC-DAD
Linearity (linear between mg/L) (correlation coefficient, expressed as r)	Primary chromatographic system: R ² = 0.9999731
	Secondary chromatographic system: R ² = 0.9999527
Precision – Repeatability Mean (100%) n = 5 (%RSD)	Primary chromatographic system: 3.92%
	Secondary chromatographic system: 1.83%
Precision – Repeatability Mean (LOQ) n = 5 (%RSD)	Primary chromatographic system: 7.35%
	Secondary chromatographic system: 6.09%
Accuracy (LOQ) n = 5 (% Recovery)	Primary chromatographic system: 103.8 (average)%
	Secondary chromatographic system: 103.5 (average)%
Accuracy (100%) n = 5 (% Recovery)	Primary chromatographic system: 76.63-123.70%
	Secondary chromatographic system: 85.26-106.05%

Interference/ Specificity	Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard phenol and peak purity. Analysis showed no overlapping of determined impurity signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.
Comment	-

Conclusion

Phenol's linearity, precision and accuracy parameters are in the acceptance range and complies with EU requirements. Results were compared to values described in Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013 - SANCO/3030/99 rev.5 22 March 2019,

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

All requirements have been described in KCP 5.2.1.1

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

Analytical methods for determination of Aclonifen impurities and relevance of CIPAC methods in AKO 600 SC were not evaluated as part of the EU review. Therefore, all relevant data is provided and considered adequate

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 Aclonifen for the generation of pre-authorization data is given in the following table.

For the detailed evaluation of additional studies it is referred to Appendix 2.

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

Component of residue definition: Aclonifen				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
Plants, plant products (Residues)	Primary	0.01 mg/kg* 0.02 mg/kg**	GC-MSD / GC-ECD	Laporte, 2003, MET2005-67
	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Class, 1999, MET2005-66
	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Ballesteros, 2005 MET2006-492
	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Davies, 2002 MET2005-65
Plants, plant products	Primary	0.01 mg/kg	LC-MS/MS	Bourgade & Diot, 2005 MET2006-493

(Residues)				
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Bourgade & Diot, 2005 MET2006-493
Animal products, food of animal origin,... (Residues)	Primary	0.01 mg/kg 0.05 mg/kg	GC-MSD / GC-ECD	Laporte, 2003, MET2005-68
	Confirmatory (if required)	-	-	Not required
Soil (Environmental fate)	Primary	0.01 mg/kg	GC-MSD /GC-ECD	Craig et al., 202 MET2005-72
	Confirmatory (if required)	-	-	Not required
Air (Exposure)	Primary	0.25 µg/m ³	GC-ECD	Heintze, 1998, MET2001-25
	Confirmatory (if required)	-	-	The lowest concentration of aclonifen in final extracts obtained from air sampling tubes is higher than in the final extracts obtained from water and confirmed by GC-MSD (0.05 µg/mL). Therefore, GC/MSD can be used as confirmatory method for air samples. An additionally validated method for confirmation is considered to be not necessary
Water, buffer solutions,... (Properties)	Primary	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001-24
	Confirmatory (if required)	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001-24
Water, buffer solutions,... (Properties)	Primary	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 2000, MET2005-72
	Confirmatory (if required)	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001-24

*LOQ in commodities with high water content and dry crops

**LOQ in commodities with high fat content and fruits with high acid content

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

Pre-authorization methods are sufficient for post-registration control and monitoring purposes. Therefore all pre-authorization and post-registration methods are described and presented in Table 5.2-3 in point KCP 5.1.2. Data is based on: DAR – Aclonifen – Volume 3 – Annex B5.

5.3.1 Analysis of the plant protection product (KCP 5.2)

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

5.3.2 Description of analytical methods for the determination of residues aclonifen (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 cur-

rent 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	Aclonifen	0.01 mg/kg	Reg. (EU) No 2021/1531
Plant, high acid content		0.02 mg/kg 0.01 mg/kg	Reg. (EU) No 2021/1531 annex II
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	Reg. (EU) No 2021/1531
Plant, high oil content		0.01 mg/kg	Reg. (EU) No 2021/1531
Plant, difficult matrices (hops, spices, tea)		0.05 mg/kg 0.03 mg/kg	Reg. (EU) No 2021/1531 annex II
Muscle	Aclonifen	0.01 mg/kg	Reg. (EU) No 2021/1531
Milk		0.01 mg/kg	Reg. (EU) No 2021/1531
Eggs		0.01 mg/kg	Reg. (EU) No 2021/1531
Fat		0.01 mg/kg	Reg. (EU) No 2021/1531
Liver, kidney		0.01 mg/kg	Reg. (EU) No 2021/1531
Soil (Ecotoxicology)	Aclonifen	0.1 µg/kg 0.006 mg/kg	Common limit ER ₅₀ <i>Brassica napus</i> : 8.28 g a.s./ha (geometric mean); DAR Addendum 12/2011, Vol. 3, B.9.9.2.1
Drinking water (Human toxicology)	Aclonifen	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Aclonifen	NOEC = 0.005 mg/L (nom)	EFSA Scientific Report (2008) 149, 1-80
Air	Aclonifen	11 µg/m ³	AOEL sys: 0.036 mg/kg bw/d
Tissue (meat or liver)	Aclonifen	According to EFSA Scientific Report (2008) 149, 1-80, not relevant, because active substance not classified as toxic or highly toxic 0.01 mg/kg	Not classified as T / T+ SANTE/2020/12830 rev.1
Body fluids		EFSA Scientific Report (2008) 149, 1-80, not relevant, because active substance not classified as toxic or highly toxic 0.01 mg/kg	Not classified as T / T+ SANTE/2020/12830 rev.1

Comments of zRMS:

According to Commission Regulation (EU) No Reg. No 283/2013 and Reg. No 284/2013 and SAN-TE/2020/12830 **rev.1 rev.2**, a fully validated analytical method for monitoring aclonifen residues in body fluids and tissue is required.

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 aclonifen in plant matrices is given in the following tables. For the detailed evaluation of additional studies it is referred to Appendix 2.

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

Component of residue definition: aclonifen				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing / EU agreed
High water content	Primary	0.01 mg/kg	GC-MSD / GC-ECD	Laporte, 2003 MET2005-67
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105060
	ILV	0.05 mg/kg	GC-MSD / GC-ECD	Class, 1999 MET2005-66
		-	-	Study for SENCIUC, 2025 S24-105060 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Ballesteros, 2005 MET2006-492
				Primary method is high specific therefore no confirmatory is needed
High acid content	Primary	0.02 mg/kg	GC-MSD / GC-ECD	Laporte, 2003 MET2005-67
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105060
	ILV	-	-	Not available
				Study for SENCIUC, 2025 S24-105060 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	Not available
		-	-	Primary method is high specific therefore no confirmatory is needed
High oil content	Primary	0.02 mg/kg	GC-MSD / GC-ECD	Laporte, 2003 MET2005-67
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105060
	ILV	-	-	Not available
		-	-	Study for SENCIUC, 2025 S24-105060 is ongoing. Will be available June/July 2025

	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Davies, 2002 MET2005-65
		-	-	Primary method is high specific therefore no confirmatory is needed
High protein/high starch content (dry)	Primary	0.01 mg/kg	GC-MSD / GC-ECD	Laporte, 2003 MET2005-67
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105060
	ILV	0.01 mg/kg	GC-MSD / GC-ECD	Class, 1999 MET2005-66
		-	-	Study for SENCIUC, 2025 S24-105060 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	0.01 mg/kg	GC-MSD / GC-ECD	Class, 1999 MET2005-66 Not required
		-	-	Primary method is high specific therefore no confirmatory is needed
Difficult (if required, depends on intended use)	Primary	0.01 mg/kg	LC-MS/MS	Bourgade & Diot, 2005 MET2006-493
	ILV	-	-	Not necessary, no intended use
	Confirmatory (if required)	0.01 mg/kg	LC-MS/MS	Bourgade & Diot, 2005 MET2006-493

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 – Aclonifen – Volume 3 – Anex B5: Methods of analysis The extraction efficiency was not assessed due to following information included in SANTE/2017/10632 Rev. 4: For renewal of product authorisations or for new product authorisations or extension of uses for which no change of the MRL is needed, the data requirements used for the latest renewal or approval should be considered. This means that no additional proof of extraction efficiency is required if it had not been required in the renewal of approval/approval procedure itself. Additionally the extraction efficiency was not assessed because according to SANTE/2017/10632 Rev. 4 if residues in metabolism studies ≥ 0.01 mg/kg do not occur extraction efficiency is not needed.

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 aclonifen in animal matrices is given in the following tables. For the detailed evaluation of additional studies it is referred to Appendix 2.

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

Component of residue definition: Aclonifen				
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	GC-ECD	Laporte, 2003 MET2005-68
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105061
	ILV	0.01 mg/kg	GC-ECD	Hausmann, 1996 MET2001-22
		-	-	Study for SENCIUC, 2025 S24-105061 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	Not available
		-	-	Primary method is high specific therefore no confirmatory is needed
Eggs	Primary	0.05 mg/kg	GC-ECD	Laporte, 2003 MET2005-68
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105061
	ILV	0.05 mg/kg	GC-ECD	Hausmann, 1996 MET2001-22
		-	-	Study for SENCIUC, 2025 S24-105061 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	Not available
		-	-	Primary method is high specific therefore no confirmatory is needed
Muscle	Primary	0.05 mg/kg	GC-ECD	Laporte, 2003 MET2005-68
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105061
	ILV	0.05 mg/kg	GC-ECD	Hausmann, 1996 MET2001-22
		-	-	Study for SENCIUC, 2025 S24-105061 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	Not available
		-	-	Primary method is high specific therefore no confirmatory is needed
Fat	Primary	0.05 mg/kg	GC-ECD	Laporte, 2003 MET2005-68
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105061
	ILV	0.05 mg/kg	GC-MSD / GC-ECD	Hausmann, 1996 MET2001-22
		-	-	Study for SENCIUC, 2025 S24-105061 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	-
		-	-	Not required

		-	-	Primary method is high specific therefore no confirmatory is needed
Kidney, liver	Primary	0.05 mg/kg	GC-MSD / GC-ECD	Laporte, 2003 MET2005-68
		0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105061
	ILV	-	-	Not available
		-	-	Study for SENCIUC, 2025 S24-105061 is ongoing. Will be available June/July 2025
	Confirmatory (if required)	-	-	Not available
		-	-	Primary method is high specific therefore no confirmatory is needed

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 Aclonifen Volume 3 Anex B5: Methods of analysis
Not required, because:	The extraction efficiency was not assessed because according to SANTE/2017/10632 Rev. 4 if residues in metabolism studies ≥ 0.01 mg/kg do not occur extraction efficiency is not needed.

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

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

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

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

Component of residue definition: aclonifen			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/kg	GC-MSD /GC-ECD	Craig et al., 202 MET2005-72
	0.01 mg/kg	LC-MS/MS	SENCIUC, 2025 S24-105064
Confirmatory	-	-	-

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

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

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

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

Component of residue definition: aclonifen				
Matrix type	Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water	Primary	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 2000, MET2005-73
		0.10 µg/L	LC-MS/MS	SENCIUC, 2025 S24-105062
	Confirmatory	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001
		-	-	Primary method is high specific therefore no confirmatory is needed
Surface water	Primary	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001-24
		0.10 µg/L	LC-MS/MS	SENCIUC, 2025 S24-105062
	Confirmatory	0.05 µg/L	GC-MSD /GC-ECD	Fuchsbichler, 1999 MET2001-24
				Primary method is high specific therefore no confirmatory is needed

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 aclonifen in air is given in the following tables. For the detailed evaluation of additional studies please refer to Appendix 2.

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

Component of residue definition: aclonifen			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.25 µg/m ³	GC-ECD	Heintze, 1998, MET2001-25
Confirmatory	Not required*		

*The lowest concentration of aclonifen in final extracts obtained from air sampling tubes is higher than in the final extracts obtained from water and confirmed by GC-MSD (0.05 µg/mL). Therefore, GC/MSD can be used as confirmatory method for air samples. An additionally validated method for confirmation is considered to be not necessary

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 aclonifen in body fluids and tissues is given in the following table. For the detailed evaluation of additional studies it is referred to Appendix 2.

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

Component of residue definition: aclonifen			
Method type	Method LOQ	Principle of method (i.e. GC-MS or HPLC-UV)	Author(s), year / missing
Primary	0.01 mg/L	LC-MS/MS	SENCIUC, 2025 S24-105063
	According to DAR — Aclonifen — Volume 3 — methods for body fluids and tissues are not required as the active substance is not classified as toxic or highly toxic however According to SANTE/2020/12830, Rev.1 24. February 2021 “Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post approval Control and Monitoring Purposes” The LOQ shall meet 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues. Higher LOQs are acceptable for analytically challenging analytes, if justified. Confirmatory methods must be submitted		
Confirmatory	Primary method is high specific therefore no confirmatory is needed		

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

5.3.2.8 Other studies/ information

Not available yet.

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	Górka I.	2023	Validation of analytical method for CHR/H/AKO 600 SC for determination of aclonifen and phenol as impurity; Study code: ICB/106/2023; Górka Iwona, MSc ICB Pharma GLP: Yes Unpublished	N	PUH “Chemiroł”
KCP 7.1	Głowiak k.	2024	Validation of an analytical method for the determination of residues of aclonifen in potato Study code: VAL/24/2023; Głowiak K. GLP: Yes Unpublished	N	PUH “Chemiroł”
KCP 5.3	Senciuc M.	2025	Development and Validation of an Analytical Method for the Determination of Aclonifen in Different Crops Study code: S24-105060 GLP: Yes Unpublished	N	PUH “Chemiroł”
KCP 5.3	Senciuc M.	2025	Development and Validation of an Analytical Method for the Determination of Aclonifen in Animal Matrices Study code: S24-105061 GLP: Yes Unpublished	N	PUH “Chemiroł”
KCP 5.3	Senciuc M.	2025	Development and Validation of an Analytical Method for the Determination of Aclonifen in Water Study code: S24-105062 GLP: Yes	N	PUH “Chemiroł”

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 5.3	Senciuc M.	2025	Development and Validation of an Analytical Method for the Determination of Aclonifen in Soil Study Code: S24-105064 GLP: Yes Unpublished	N	PUH "Chemiro"
KCP 5.3	Senciuc M.	2025	Development and Validation of an Analytical Method for the Determination of Aclonifen in Body Fluids Study Code: S24-105063 GLP: Yes Unpublished	N	PUH "Chemiro"

Not available yet

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 5.1.2 KCP 5.2	Bourgade, C. and Diot, R.	2005	Analytical method 00950 for the determination of residues of aclonifen in/on plants by HPLC-MS/MS. MR-111/05; M-262314-01-1 GLP, unpublished MET2006-493	N	BAY4

KCP 5.1.2 KCP 5.2	Class, T.	1999	Aclonifen: Assessment and Validation of the Multi-Residue Enforcement Method DFG S19 with Modified Extraction for the Determination of Residues of Aclonifen in Cereals (Wheat, Grain and Straw, Barley Grain and Straw, Corn Grain and Silage). R007421 GLP, unpublished MET2005-66	N	BAY4
KCP 5.1.2 KCP 5.2	Ballesteros, C.	2005	Analytical method 00889 for the determination of residues of aclonifen in/on potatoe by GC/MS C047094; MR-103/04 GLP, unpublished MET2006-492	N	BAY4
KCP 5.1.2 KCP 5.2	Davies, P	2002	Residues at harvest in sunflowers European Union (Northern zone) 2001 Aclonifen water miscible suspension concentrate (SC) 49.59 % w/w (600 g/L) Code: AE F068300 00 SC50A202. C021765 GLP, unpublished MET2005-65	N	BAY4
KCP 5.1.2 KCP 5.2	Hausmann, S.	1996	Analytical Enforcement Method (DFG S19) for the Determination of Aclonifen in Food-stuff of Animal Origin by GC/ECD and/or GC/MS. PTRL Study P 212 G R007388 ! R&D/CRLD/AN/9616581 GLP, unpublished MET2001-22	Y	BAY4
KCP 5.1.2 KCP 5.2	Laporte, F.	2003	Validation of the multi-residue enforcement method DFG S19 (extended revision) for the determination of aclonifen in animal matrices. Code: AE F068300 C031065 GLP, unpublished MET2005-68	Y	BAY4
KCP 5.1.2 KCP 5.2	Laporte, F.	2003	Validation of the multi-residue enforcement method DFG S19 (extended revision) for the determination of aclonifen in plant matrices. Code: AE F068300 C031064 GLP, unpublished MET2005-67	N	BAY4

KCP 5.1.2 KCP 5.2	Heintze, A.	1998	Developing an Analytical Method for the Determination of Aclonifen in Air around 35 °C and 80 % Humidity. Final Report 98228/01-CMLU GLP, unpublished MET2001-25	N	BAY4
KCP 5.1.2 KCP 5.2	Fuchsbichler, G.	2000	Validation of an Analytical Method for the Determination of Aclonifen in Drinking water. R007422 GLP, unpublished MET2005-73	N	BAY4
KCP 5.1.2 KCP 5.2	Fuchsbichler, G.	1999	Validation of an Analytical Method for the Determination of Aclonifen in Surface Water (River, Pond). HVA 27/99 R008576 GLP, unpublished MET2001-24	N	BAY4
KCP 5.1.2 KCP 5.2	Craig, A., Pirie, L.M., Doran, A.M. and McGuire, G.M	2002	Aclonifen: Validation of an analytical method to determine residues of aclonifen in soil. C020037 GLP, unpublished MET2005-72	N	BAY4

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for aclonifen

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

No new or additional studies have been submitted

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

~~No new or additional studies have been submitted~~
New studies have been submitted.

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~~
New studies have been submitted.

Comments of zRMS:	The analytical method code: S24-105060 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830, rev. 2 The results of analytical method validation confirm that this method is suitable for determination of aclonifen in different crops apple fruit, grape (fruit), wheat (grain), oilseed rape seed (seeds) The method is successfully validated and accepted.
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Reference: KCP 5.3

Report Development and Validation of an Analytical Method for the Determination of Aclonifen in Different Crops, Study Code: S24-105060, SENCIUC M.: 2025

GLP: Yes

Deviations: No

Duplication (if vertebrate study) No

Acceptability: Yes

Summary:

Item	Activity, Result, Assessment
Study Scope	Validation of an analytical method according to guidance document(s) SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analyte	Aclonifen

Matrices	Apple fruit, grape (fruit), wheat (grain), oilseed rape seed (seeds)											
Method Reference	Multi-residue method QuEChERS											
LOQ	0.01 mg/kg (lowest validated fortification level)											
LOD	30 % of the LOQ (lowest calibration standard)											
Principle of the Analytical Procedure	Homogenisation: Dry ice, cutting mill Extraction: Acetonitrile and addition of water (if needed), shaken Liquid-liquid Partition: citrate buffer salt tube, shaking, centrifugation Clean-up: Dispersive SPE with primary/secondary amine (PSA) Additional clean-up for oilseed rape seed and wheat grain: freezing Dilutions: Acetonitrile/water (50/50, v/v) Sample concentration in final extract: 0.01 g sample per mL of extract Quantification: LC-MS/MS											
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.											
Matrix Effects on Analyte Detection	Insignificant (≤ 20 %) for apple and wheat grain. Significant ($>20\%$) for grapes and rape seed.											
Calibration	Matrix-matched standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.030 ng/mL to 3.0 ng/mL Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a (diluted) sample extract The validated range does not exceed two (2) orders of magnitude											
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed Correlation coefficients (R) ≥ 0.99											
Accuracy and Precision	Five (5) fortifications at 0.01 mg/kg (LOQ) Five (5) fortifications at 0.1 mg/kg (10x LOQ) Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2: <table><tr><td>Concentration Level (mg/kg)</td><td>Range of Mean Recoveries (%)</td><td>Precision, Rel. Std. Dev. (%)</td></tr><tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr><tr><td>$> 0.01 - \leq 0.1$</td><td>70 - 120</td><td>≤ 20</td></tr></table>			Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)	≤ 0.01	60 - 120	≤ 30	$> 0.01 - \leq 0.1$	70 - 120	≤ 20
Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)										
≤ 0.01	60 - 120	≤ 30										
$> 0.01 - \leq 0.1$	70 - 120	≤ 20										
Stability of Analyte(s) in Standard Solutions	Within ± 10 % for 79 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark.											
Stability of Analyte in Sample Extracts	Recoveries within 70 % - 120 % in all matrix extracts for at least 13 days when stored at typically 1 °C to 10 °C in the dark.											
Conclusion	The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring											

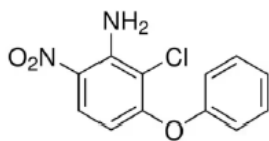
Materials and methods:

Test/Reference items

Common name: Aclonifen

Chemical name: 2-chloro-6-nitro-3-phenoxybenzenamine

Chemical structure:



CAS number: 74070-46-5

Empirical formula: C₁₂H₉ClN₂O₃

Molecular weight: 264.7 g/mol

Supplier Sigma Aldrich - Purity analysed: 99.8 %

Date of certificate (Quality Release Date): 15 Jun 2022; Lot number BCCH7675

Date of receipt at Test Facility: 10 Oct 0224; Expiration date May 2027

Internal Tracking Number R08473

Storage condition at Test Facility: Ambient

Reagents and Materials

- Acetonitrile, HPLC grade, VWR
- LC-MS Formic acid, , LC-MS grade, Promochem
- LC-MS Acetonitrile, (ACN) LC-MS grade
- LC-MS Water, Merck, LC-MS grade (H₂O)
- Milipore Water (supply at Test Facility)
- Dispersive SPE Citrate Extraction Tube (Supelco, Art.No. 55227-U)
- Dispersive SPE PSA SPE Clean-up Tube 1 (Supelco, Art.No. 55228-U)
- Supelclean PSA, Supelco, (52738-U)

Instrumentation and Apparatus

- Common laboratory glassware
- Analytical Balance, XS205DU (Mettler Toledo)
- Balance, Sartorius ED 2202S-CW (Mettler Toledo)
- Knife mill, GM 300, Retsch
- Robot Coupe R20, Robot Coupe
- Vortex mixer REAX top, Heidolph
- Horizontal shaker HS 501 D, IKA
- Ultrasonic bath Transonic 460, Elma Hans Schmidbauer
- Centrifuge Rotanta 460, Hettich

Matrix Type(s), Sample Origin, Preparation and Storage

Matrix Type(s)	Preparation/Storage	Origin
Apple fruit, Grape (fruit), Wheat (grain), Oilseed Rape Seed (seeds)	Apple fruit: the sample material was thoroughly homogenised in a knife mill. Grape (fruit): After removal of stems the sample material was thoroughly homogenised in a knife mill with dry ice. Wheat grain and oilseed rape seeds: the sample material was thoroughly homogenised in a knife mill with dry ice. Thereafter, apple and grapes were stored frozen at typically $\leq -18^{\circ}\text{C}$. Wheat grain and oilseed rape seed were stored at ambient temperature.	Purchased commercially.

Method Summary

In brief, the samples were extracted with acetonitrile, after addition of water (if needed). Then the magnesium sulfate, sodium chloride and buffering citrate salt mixture was added and shaken intensively followed by centrifugation for phase separation. Clean-up of the apple, grape, wheat grain and oilseed rape seeds extract was performed by dispersive SPE with primary/secondary amine (PSA). An additionally clean-up for wheat grain and oilseed rape seeds was the freezing step. Finally the extract of all matrices was diluted with water/acetonitrile (1/1, v/v). Quantification was performed by use of LC MS/MS.

Selectivity and Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 265 $\rightarrow$ 248 and m/z 265 $\rightarrow$ 182). Due to enhanced sensitivity mass transition m/z 265 $\rightarrow$ 248 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation. A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions the samples showed no significant interference that would correspond to 30 % of LOQ at the retention time of the analyte in any investigated matrix (apple fruit, grape fruit, wheat grain and oilseed rape seeds), therefore showing that the method is highly specific. Blank correction was not performed.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing peak areas of matrix-matched standards (at least 90 % matrix amount) with solvent standards at the same nominal concentrations.

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} / (A_{\text{Solv-Std}}))] - 100$
$A_{\text{Solv-Std}}$	Peak area of solvent standard
$A_{\text{Matrix-Std}}$	Peak area of matrix-matched standard

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Aclonifen (%)	
		Proposed for Quantification (m/z 265→248)	Proposed for Confirmation (m/z 265→182)
Apple	1.0 (10x LOQ)	(+) 4	(+) 6
	0.10 (LOQ)	(-) 2.6	(+) 2
Grapes	1.0 (10x LOQ)	(+) 29	(+) 32
Wheat grain	1.0 (10x LOQ)	(+) 8	(+) 12
	0.10 (LOQ)	(+) 5	(+) 9
Oilseed rape seeds	1.0 (10x LOQ)	(-) 29	(-) 28
	0.10 (LOQ)	(-) 31	(-) 32

Matrix effects were $< \pm 20$ % and deemed to be insignificant apple and wheat grain, and significant ($>20\%$) for grape and rape seeds. Matrix-matched standard solutions were used for quantification throughout the study.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.030 ng/mL to 3.0 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. The calibration curve does not exceed two (2) orders of magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear; the regression residuals were randomly distributed on visual inspection. Furthermore, correlation coefficients (R) were ≥ 0.99 .

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was distributed over the whole analytical acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/kg was confirmed in all matrices. The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for all matrices, which is 30 % of the LOQ.

Accuracy and Precision

Aclonifen							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition m/z 265→248 (Proposed for Quantification)							
Apple	0.01	94; 101; 103; 103; 94	99	5	5	95	6
	0.1	85; 89; 90; 93; 94	90	4	5		
Grape	0.01	100; 94; 98; 101; 101	99	3	5	101	3
	0.1	104; 103; 102; 103; 105	103	1	5		
Wheat grain	0.01	96; 95; 105; 89; 97	96	6	5	95	5
	0.1	94; 93; 97; 89; 93	93	3	5		
Oilseed rape seeds	0.01	80; 86; 82; 78; 76	80	5	5	80	5
	0.1	76; 83; 83; 78; 76	79	5	5		

Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition m/z 265→182 (Proposed for Confirmation)							
Apple	0.01	95; 103; 98; 96; 92	97	4	5	94	5
	0.1	86; 91; 90; 92; 94	91	4	5		
Grape	0.01	102; 93; 99; 95; 101	98	4	5	101	4
	0.1	103; 101; 104; 103; 105	103	1	5		
Wheat grain	0.01	102; 97; 101; 93; 95	98	4	5	95	4
	0.1	95; 95; 94; 90; 91	93	3	5		
Oilseed rape seeds	0.01	78; 86; 82; 79; 75	80	5	5	79	5
	0.1	76; 82; 84; 76; 75	78	5	5		

Stability of Standard Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5).

Analyte	Solvent	Concentration (µg/mL)	Concentration after Dilution for Measurement (ng/mL)	Storage Period (Days)	Difference (in %) of stored stock solution compared to freshly prepared stock solution
Aclonifen (m/z 265→248)	Acetonitrile	1000	10	79	- 1.8

The mean peak area of the stock standard solution was within ± 10 % of the mean peak area of the freshly prepared solution indicating that stock solution is stable when stored at 1 °C to 10 °C in the dark for 79 days. Fortification and calibration solutions were prepared in the same solvent as stock solutions and thus did not require an extra testing.

Stability of Final Extracts

Following the first analysis final extracts of samples fortified at the 10x LOQ level together with one (1)

control sample extract were stored at typically 1 °C to 10 °C in the dark. After storage the final extracts were analysed against freshly prepared calibration standards. One (1) mass transition was evaluated. The results obtained are summarised in the table below.

Matrix	Fortification level (mg/kg)	Days of storage *	Mean recovery Injection (n = 2**) after Storage (%)
Apple (m/z 265→248)	0.10	27	90
Grapes (m/z 265→248)	0.10	27	93
Wheat Grain (m/z 265→248)	0.10	27	90
Oilseed Rape Seeds (m/z 265→248)	0.10	13	78

Conclusion

The method was successfully validated for the determination of aclonifen in/on apple fruit, grape fruit, oilseed rape seed and wheat grain matrices from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reference List

- EN 15662:2018: "Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method".
- Literature: "Food Composition and Nutrition Tables", Souci, Fachmann, Kraut, 8th revised and completed edition.

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

No new or additional studies have been submitted
New studies have been submitted.

Comments of zRMS:	The analytical method code: S24-105061 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830, rev. 2 The results of analytical method validation confirm that this method is suitable for determination of aclonifen in Animal Matrices (Bovine (whole milk), Poultry's eggs, Bovine (fat), Bovine (liver), Bovine (muscle)). The method is successfully validated and accepted.
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Reference: KCP 5.3

Report Development and Validation of an Analytical Method for the Determination of Aclonifen in Animal Matrices Study Code: S24-105061, SENCIUC M.; 2025

GLP: Yes

Deviations: No

Duplication (if vertebrate study) No

Acceptability: Yes

Summary:

Item	Activity, Result, Assessment		
Study Scope	Validation of an analytical method according to guidance document(s) SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring		
Analyte	Aclonifen		
Matrices	Bovine (whole milk), Poultry's eggs, Bovine (fat), Bovine (liver), Bovine (muscle)		
Method Reference	Multi-residue method QuEChERS		
LOQ	0.01 mg/kg (lowest validated fortification level)		
LOD	30 % of the LOQ (lowest calibration standard)		
Principle of the Analytical Procedure	Homogenisation of Bovine (liver) and Bovine (muscle): Dry ice, knife mill Homogenisation of Bovine (whole milk): Shaken prior analysis Homogenisation of Poultry's eggs: De-shelled, yolk and egg whites were quilted Homogenisation of Bovine (fat): rendered Extraction: Addition of water if necessary and acetonitrile shaking on a shaker Liquid-liquid Partition: citrate buffer salt tube, shaking, centrifugation Clean-up step: PSA SPE clean tube (not nessersary for bovine (fat)), shaking, centrifugation Dilution: Acetonitrile/Water (50/50, v/v) Quantification: LC-MS/MS		
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.		
Matrix Effects on Analyte Detection	Significant (≥ 20 %) for Bovine (fat) Insignificant (< 20 %) Bovine (whole milk), Poultry's eggs, Bovine (liver) and Bovine (muscle)		
Calibration	Matrix-matched calibration standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.030 ng/mL to 3.0 ng/mL Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in sample extract The validated range does not exceed two (2) orders of magnitude		
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed Correlation coefficients (R) ≥ 0.99		
Accuracy and Precision	Five (5) fortifications at 0.01 mg/kg (LOQ) Five (5) fortifications at 0.1 mg/kg (10x LOQ)		
	Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)
	≤ 0.01	60 - 120	≤ 30
	$> 0.01 - \leq 0.1$	70 - 120	≤ 20

Stability of Analyte(s) in Standard Solutions	Within $\pm 10\%$ for 79 days when prepared in acetonitrile and stored at typically $1\text{ }^{\circ}\text{C}$ to $10\text{ }^{\circ}\text{C}$ in the dark. Calibration solutions in acetonitrile/water 1/1, v/v (used only to determine the matrix-effect) were freshly prepared. For this reason these solutions were not checked for stability.
Stability of Analyte in Sample Extracts	Recoveries within 70% - 120% in all matrix extracts for at least 18 days when stored at typically $1\text{ }^{\circ}\text{C}$ to $10\text{ }^{\circ}\text{C}$ in the dark.
Conclusion	The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

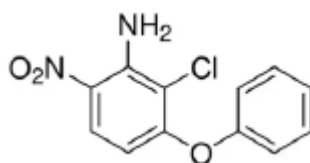
Materials and methods

Test and reference items

Common name: Aclonifen

Chemical name: 2-chloro-6-nitro-3-phenoxybenzenamine

Chemical structure



CAS number: 74070-46-5

Empirical formula: $\text{C}_{12}\text{H}_9\text{ClN}_2\text{O}_3$

Molecular weight: 264.7 g/mol

Supplier Sigma Aldrich - Purity analysed: 99.8%

Date of certificate (Quality Release Date) 15 Jun 2022; Lot number BCCH7675

Date of receipt at Test Facility - 10 Oct 2024; Expiration date May 2027

Internal Tracking Number: R08473

Storage conditions at Test Facility: Ambient

Reagents and Materials

- Acetonitrile, HPLC grade (VWR, Art.No. 20060.320)
- LC-MS Formic acid, Promochem, LC-MS grade (FA)
- LC-MS Acetonitrile, Merck, LC-MS grade (ACN)
- LC-MS Water, Merck, LC-MS grade (H_2O)
- Dispersive SPE Citrate Extraction Tube (Supelco, Art.No. 55227-U)
- Dispersive SPE PSA SPE Clean-up Tube 1 (Supelco, Art.No. 55228-U)
- Milipore Water (supply at Test Facility)

Instrumentation and Apparatus

- Common laboratory glassware
- Analytical Balance, XS205DU (Mettler Toledo)
- Balance, Sartorius ED 2202S-CW (Mettler Toledo)
- Knife mill, GM 300, Retsch
- Vortex mixer REAX top, Heidolph
- Horizontal shaker HS 501 D, IKA
- Ultrasonic bath Transonic 460, Elma Hans Schmidbauer
- Centrifuge Rotanta 460, Hettich

Matrix Type(s), Sample Origin, Preparation and Storage

Matrix Type(s)	Preparation	Origin
Bovine (whole milk)	The sample material was shaken prior to taking aliquots for analysis.	Local Supermarket
Poultry's eggs	The sample material was de-shelled and the yolk and egg whites were quiled. Aliquots of frozen sample material were taken for analysis.	Local Supermarket
Bovine (fat)	The sample material was rendered. Aliquots of frozen sample material were taken for analysis.	Local Slaughterhouse
Bovine (liver)	The sample material was thoroughly homogenised in a knife mill with dry ice. Aliquots of frozen sample material were taken for analysis.	Local Supermarket
Bovine (muscle)	The sample material was thoroughly homogenised in a knife mill with dry ice. Aliquots of frozen sample material were taken for analysis.	Local Supermarket

Method Summary

Item	Activity
Principle of the Analytical Procedure	Homogenisation of Bovine (liver) and Bovine (muscle): Dry ice, knife mill Homogenisation of Bovine (whole milk): Shaken prior analysis Homogenisation of Poultry's eggs: De-shelled, yolk and egg whites were quiled Homogenisation of Bovine (fat): Rendered Extraction: Addition of water (if necessary) and acetonitrile; shaking on a shaker. Liquid-liquid Partition: citrate buffer salt tube, shaking, centrifugation Clean-up step (not necessary for bovine (fat)): PSA SPE clean tube, shaking, centrifugation Dilution: Acetonitrile/Water (50/50, v/v) Quantification: LC-MS/MS

Selectivity and Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 265→248 and m/z 265→182). Due to enhanced sensitivity mass transition m/z 265→248 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation. A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions the samples showed no significant interference that would correspond to 30 % of LOQ at the retention time of the analyte in any investigated matrix bovine (whole milk), poultry's eggs, bovine (fat), bovine (liver) and bovine (muscle), therefore showing that the method is highly specific. Blank correction was not performed.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing mean peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at nominal concentrations. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}}) / (A_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Peak area of solvent standard
$A_{\text{Matrix-Std}}$	Peak area of matrix-matched standard

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Aclonifen (%)	
		Proposed for Quantification (m/z 265→248)	Proposed for Confirmation (m/z 265→182)
Bovine (whole milk)	1.0 (10xLOQ)	(+) 0.1	(-) 2.6
	0.10 (LOQ)	(+) 1.1	(+) 2.3
Poultry's eggs	1.0 (10xLOQ)	(-) 15	(-) 13
	0.10 (LOQ)	(-) 18	(-) 13
Bovine (fat)	1.0 (10xLOQ)	(-) 22	(-) 22
	0.10 (LOQ)	(-) 26	(-) 27
Bovine (liver)	1.0 (10xLOQ)	(-) 12	(-) 11
	0.10 (LOQ)	(-) 12	(-) 13
Bovine (muscle)	1.0 (10xLOQ)	(-) 19	(-) 18
	0.10 (LOQ)	(-) 1.5	(-) 5.3

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.030 ng/mL to 3.0 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99 .

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was distributed over the whole analytical acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/kg was confirmed for aclonifen in bovine (whole milk), poultry's eggs, bovine (fat), bovine (liver) and bovine (muscle). The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg for all matrices, which is 30 % of the LOQ.

Accuracy and Precision

Aclonifen							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition m/z 265→248 (Proposed for Quantification)							
Bovine (whole milk)	0.01	93, 88, 90, 85, 100	91	6	5	94	6
	0.1	92, 100, 96, 99, 96	97	3	5		
Poultry's eggs	0.01	81, 90, 91, 90, 90	88	5	5	88	4
	0.1	92, 82, 89, 89, 85	87	4	5		
Bovine (fat)	0.01	73, 75, 75, 75, 70	74	3	5	75	3
	0.1	75, 77, 78, 75, 74	76	2	5		
Bovine (liver)	0.01	88, 95, 97, 11*, 100	95	5	4	93	5
	0.1	87, 90, 94, 90, 94	91	3	5		
Bovine (muscle)	0.01	99, 93, 91, 89, 92	93	4	5	94	4
	0.1	100, 92, 94, 91, 98	95	4	5		

Aclonifen							
Matrix	Fortification Level (mg/kg)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition m/z 265→182 (Proposed for Confirmation)							
Bovine (whole milk)	0.01	94, 91, 93, 89, 100	93	4	5	95	4
	0.1	91, 99, 98, 98, 98	97	3	5		
Poultry's eggs	0.01	82, 89, 85, 90, 86	86	4	5	86	3
	0.1	90, 84, 86, 86, 84	86	3	5		
Bovine (fat)	0.01	77, 74, 83, 77, 71	76	6	5	76	4
	0.1	73, 77, 77, 76, 75	75	2	5		
Bovine (liver)	0.01	90, 96, 93, 11*, 99	94	4	4	92	4
	0.1	88, 87, 94, 91, 93	91	3	5		
Bovine (muscle)	0.01	96, 96, 91, 89, 95	93	4	5	95	4
	0.1	102, 94, 94, 92, 99	96	4	5		

Concentration Level (mg/kg)	Range of Mean Recoveries (%)	Precision, Rel. Std. Dev. (%)
≤ 0.01	60 – 120	≤ 30
> 0.01 - ≤ 0.1	70 - 120	≤ 20

Stability of Standard Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5).

Analyte	Solvent	Concentration (µg/mL)	Concentration after Dilution for Measurement (ng/mL)	Storage Period (Days)	Difference (in %) of stored stock solution compared to freshly prepared stock solution
Aclonifen (m/z 265→248)	Acetonitrile	1000	10	79	- 1.8

Stability of Final Extracts

Matrix	Analyte	Fortification level (mg/kg)	Days of storage *	Mean recovery Injection (n = 5) after Storage (%)	Rel. Std. Dev. (n = 5) (%)
Bovine (whole milk)	Aclonifen (m/z 265→248)	0.10	31	98	9
Poultry's eggs	Aclonifen (m/z 265→248)	0.10	30	83	8
Bovine (fat)	Aclonifen (m/z 265→248)	0.10	18	82	4
Bovine (liver)	Aclonifen (m/z 265→248)	0.10	31	99	9
Bovine (muscle)	Aclonifen (m/z 265→248)	0.10	30	94	4

Conclusion

The method was successfully validated for the determination of aclonifen in bovine (whole milk), poultry's eggs, bovine (fat), bovine (liver) and bovine (muscle) from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reference List

- EN 15662:2018: "Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method".

- Literature: "Lebensmitteltabelle für die Praxis", Der kleine Souci, Fachmann, Kraut, Herausgeber Deutschen Forschungsanstalt für Lebensmittelchemie, 3. Auflage, ISBN: 3-8047-2037-4.

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

~~No new or additional studies have been submitted~~

New studies have been submitted.

Comments of zRMS:	The analytical method code: S24-105064 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830, rev. 2 The results of analytical method validation confirm that this method is suitable for determination of aclonifen in Soil. The method is successfully validated and accepted.
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Reference:

KCP 5.3

Report	Development and Validation of an Analytical Method for the Determination of Aclonifen in Soil; Study Code: S24-105064, SENCIUC M.; 2025
GLP:	Yes
Deviations:	No
Acceptability:	Yes
Duplication (if vertebrate study)	No

Summary

Item	Activity, Result, Assessment
Study Scope	Validation of an analytical method according to guidance document(s) SAN-TE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analyte	Aclonifen
Matrices	Soil
Method Reference	Based on Multi-residue method QuEChER
LOQ	0.01 mg/kg (based on dry weigh)
LOD	30 % of the LOQ (lowest calibration standard))
Principle of the Analytical Procedure	Extraction: Acetonitrile and addition of water, shaken Liquid-liquid partition: citrate buffer salt tube, shaking, centrifugation Clean-up: Dispersive PSA SPE clean tube shaking, centrifugation Dilutions: Acetonitrile/water (50/50, v/v) Sample concentration in final extract: 0.01 g sample per mL of extract Quantification: LC-MS/MS
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.
Matrix Effects on Analyte Detection	Insignificant ($\leq 20\%$)
Calibration	Matrix-matched standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.030 ng/mL to 3.0 ng/mL Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a (diluted) sample extract The validated range does not exceed two (2) orders of magnitude
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed Correlation coefficients ($R \geq 0.99$)
Accuracy and Precision	n Five (5) fortifications at 0.01 mg/kg (LOQ), based on dry weight Five (5) fortifications at 0.1 mg/kg (10x LOQ), based on dry weight Mean recoveries for both (2) evaluated (2) mass transitions are within 70 % - 120 % with relative standard deviations $\leq 20\%$ and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2
Stability of Analyte(s) in Standard Solutions	Within $\pm 10\%$ for 79 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark.
Stability of Analyte in Sample Extracts	Recoveries within 70 % - 120 % for at least 9 days when stored at typically 1 °C to 10 °C in the dark.
Conclusion	The method was found to be valid according to the guidance document SAN-

TE/2020/12830, rev. 2 for risk assessment and/or monitoring

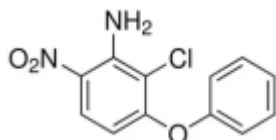
Materials and Methods

Test / Reference Item(s)

Common name: Aclonifen

Chemical name: 2-chloro-6-nitro-3-phenoxybenzenamine

Chemical structure:



CAS number: 74070-46-5

Empirical formula: C₁₂H₉ClN₂O₃

Molecular weight: 264.7 g/mol

Supplier: Sigma Aldrich; Purity analysed: 99.8 %

Date of certificate (Quality Release Date): 15 Jun 2022; Lot number BCCH7675

Date of receipt at Test Facility: 10 Oct 2024

Expiration date: May 2027; Internal Tracking Number: R08473

Storage conditions at Test Facility: Ambient

Reagents and Materials

- Acetonitrile, HPLC grade, VWR
- LC-MS Formic acid, , LC-MS grade, Promochem
- LC-MS Acetonitrile, (ACN) LC-MS grade
- LC-MS Water, Merck, LC-MS grade (H₂O)
- Milipore Water (supply at Test Facility)
- Dispersive SPE Citrate Extraction Tube (Supelco, Art.No. 55227-U)
- Dispersive SPE PSA SPE Clean-up Tube 1 (Supelco, Art.No. 55228-U)

Instrumentation and Apparatus

- Common laboratory glassware
- Analytical Balance, XS205DU (Mettler Toledo)
- Balance, ENTRIS220I-IS (Sartorius)
- Vortex mixer REAX top, Heidolph
- Horizontal shaker HS 501 D, IKA
- Ultrasonic bath Transonic 460, Elma Hans Schmidbauer
- Centrifuge Rotanta 460, Hettich

Matrix Type(s), Sample Origin, Preparation and Storage

Matrix Type(s)	Preparation (and Origin)
Soil (LUFA 2.2)	Certified standard soil was obtained commercially (LUFA). A certificate of analysis is included in the final report. The moisture content was determined in accordance with the respective SOP.

Method Summary

In brief, the samples were extracted with acetonitrile, after addition of water. Then the magnesium sulfate, sodium chloride and buffering citrate salt mixture was added and shaken intensively followed by centrifugation for phase separation. Clean-up of the sample extract was performed by dispersive SPE with primary/secondary amine (PSA). Final, the extract was diluted with water/acetonitrile (1/1, v/v). Quantification was performed by use of LC MS/MS.

Selectivity and Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 265→248 and m/z 265→182). Due to enhanced sensitivity mass transition m/z 265→248 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation. A reagent blank and two (2) control samples per matrix/analyte were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions the samples showed no significant interference that would correspond to 30 % of LOQ at the retention time of the analyte, therefore showing that the method is highly specific. Blank correction was not performed.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at the same nominal concentrations. Matrix effects were calculated as follows:

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}} / (A_{\text{Solv-Std}}))] - 100$		
$A_{\text{Solv-Std}}$	Peak area of solvent standard		
$A_{\text{Matrix-Std}}$	Peak area of matrix-matched standard		

Matrix	Standard Concentration (ng/mL)	Matrix Effect for Aclonifen (%)	
		Proposed for Quantification (m/z 265→248)	Proposed for Confirmation (m/z 265→182)
Soil	1.0 (10x LOQ)	(+) 2.1*	(+) 1*

Matrix effects were $< \pm 20$ % and deemed to be insignificant. Matrix-matched standard solutions were used for quantification throughout the study.

Calibration

The linearity of the detector response was demonstrated by determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.030 ng/mL to 3.0 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/kg to 0.30 mg/kg and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. The calibration curve does not exceed two (2)

orders of magnitude.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was distributed over the whole analytical acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/kg was confirmed in soil. The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/kg, which is 30 % of the LOQ. As can be seen from representative chromatograms in Appendix D the chromatographic peaks at the LOD were equivalent to three times or more than the background noise.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation).

Aclonifen							
Matrix	Fortification Level (mg/kg, based on dry weight)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition <i>m/z</i> 265→248 (Proposed for Quantification)							
Soil	0.01	92.1; 93.5; 92.1; 91.4; 92.1	94.0	2.3	5	93.1	1.9
	0.1	96.9; 95.5; 92.7; 92.0; 92.7	92.2	0.8	5		
Mass Transition <i>m/z</i> 265→182 (Proposed for Confirmation)							
Soil	0.01	92.4; 91.0; 92.2; 88.5; 94.7	92.1	1.8	5	92.0	2.0
	0.1	93.7; 93.3; 92.7; 89.6; 91.4	91.8	2.5	5		

All mean recovery values at fortification levels of 0.01 mg/kg and 0.1 mg/kg for two (2) mass transitions are within 70 % - 120 % with relative standard deviations $\leq$ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2

Stability of Standard Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5).

Analyte	Solvent	Concentration (μ g/mL)	Concentration after Dilution for Measurement (ng/mL)	Storage Period (Days)	Difference (in %) of stored stock solution compared to freshly prepared stock solution
Aclonifen (m/z 265→248)	Acetonitrile	1000	10	79	- 1.8

The mean peak area of the stock standard solution was within $\pm$ 10 % of the mean peak area of the freshly prepared solution indicating that stock solution is stable when stored at 1 °C to 10 °C in the dark for 79 days.

Stability of Final Extracts

Following the first analysis final extracts of samples fortified at the 10x LOQ level together with one (1) control sample extract were stored at typically 1 °C to 10 °C in the dark. After storage the final extracts were analysed against freshly prepared matrix-matched calibration solutions. One (1) mass transition was evaluated. The results obtained are summarised in the table below.

Matrix	Fortification level (mg/kg)	Days of storage	Mean recovery Injection (n = 5) after Storage (%)	Relative Standard Deviation Injection (n = 5) after Storage (%)
Soil (m/z 265→248)	0.10	9	94.4	3.1

The mean recovery value of the stored extracts were in the range of 70 % - 120 % with a relative standard deviation ≤ 20 % when analysed against freshly prepared calibration standards. Therefore, extracts are considered to be stable when stored at typically 1 °C to 10 °C for at least 9 days in the dark.

Storage Stability

Storage stability testing with fortified or incurred samples was not conducted as part of this study.

Conclusion

The method was successfully validated for the determination of aclonifen in soil from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reference List

- EN 15662:2018: "Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method “.

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

~~No new or additional studies have been submitted~~

New studies have been submitted.

Comments of zRMS:	The analytical method code: S24-105062 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830, rev. 2 The results of analytical method validation confirm that this method is suitable for determination of aclonifen in Water (drinking water and surface water). The method is successfully validated and accepted.
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Reference:

KCP 5.3

Report

Development and Validation of an Analytical Method for the Determination of Aclonifen in Water, Study Code: S24-105062, SENCIUC M.; 2025

GLP: Yes
Duplication (if vertebrate study) No
Acceptability: Yes

Summary:

Item	Activity, Result, Assessment
Study Scope	Validation of an analytical method according to guidance document(s) SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analyte	Aclonifen
Matrices	Drinking water and Surface water
Method Reference	Multi-residue method QuEChERS
LOQ	0.1 µg/L (lowest validated fortification level)
LOD	30 % of the LOQ (lowest calibration standard)
Principle of the Analytical Procedure	Homogenisation: reach room temperature, shaken settle of particulates Extraction: Addition of acetonitrile shaking on a shaker Liquid-liquid Partition: citrate buffer salt tube, shaking, centrifugation Clean-up step: PSA SPE clean tube shaking, centrifugation Dilution: Acetonitrile/Water (50/50, v/v) Quantification: LC-MS/MS
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.
Matrix Effects on Analyte Detection	Insignificant (< 20 %) for Drinking water and Surface water
Calibration	Matrix-matched calibration standards A minimum of five (5) concentration levels Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.015 ng/mL to 1.5 ng/mL Corresponding mass fraction range: 0.03 µg/L to 3.0 µg/L Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in sample extract The validated range does not exceed two (2) orders of magnitude
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed Correlation coefficients (R) ≥ 0.99
Accuracy and Precision	Five (5) fortifications at 0.1 µg/L (LOQ) Five (5) fortifications at 1.0 µg/L (10x LOQ) Recovery values should be between 70-120%. Results should have a relative standard deviation of ≤ 20 %.
Stability of Analyte(s) in Standard Solutions	Within ± 10 % for 169 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark. Calibration solutions in acetonitrile/water 1/1, v/v (used only to determine the matrix-effect) were freshly prepared. For this reason these solutions were not checked for stability.
Stability of Analyte in Sample Extracts	Recoveries within 70 % - 120 % in all matrix extracts for at least 9 days when stored at typically 1 °C to 10 °C in the dark.
Conclusion	The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

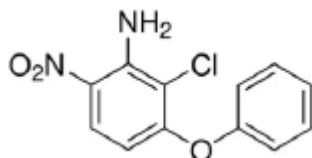
Materials and methods

Test and reference items

Common name: Aclonifen

Chemical name: 2-chloro-6-nitro-3-phenoxybenzenamine

Chemical structure



CAS number: 74070-46-5

Empirical formula: C₁₂H₉ClN₂O₃

Molecular weight: 264.7 g/mol

Supplier Sigma Aldrich - Purity analysed: 99.8 %

Date of certificate (Quality Release Date) 15 Jun 2022; Lot number BCCH7675

Date of receipt at Test Facility - 10 Oct 2024; Expiration date May 2027

Internal Tracking Number: R08473

Storage conditions at Test Facility: Ambient

Reagents and Materials

- Acetonitrile, HPLC grade (VWR, Art.No. 20060.320)
- LC-MS Formic acid, Promochem, LC-MS grade (FA)
- LC-MS Acetonitrile, Merck, LC-MS grade (ACN)
- LC-MS Water, Merck, LC-MS grade (H₂O)
- Dispersive SPE Citrate Extraction Tube (Supelco, Art.No. 55227-U)
- Dispersive SPE PSA SPE Clean-up Tube 1 (Supelco, Art.No. 55228-U)
- Milipore Water (supply at Test Facility)

Instrumentation and Apparatus

- Common laboratory glassware
- Analytical Balance, XS205DU (Mettler Toledo)
- Vortex mixer REAX top, Heidolph
- Horizontal shaker HS 501 D, IKA
- Ultrasonic bath (for preparation of stock solution)
- Centrifuge Rotanta 460, Hettich

Matrix Type(s), Sample Origin, Preparation

Matrix Type(s)	Preparation	Origin
Drinking water [2]	The sample material was allowed to reach room temperature and shaken thoroughly. After shaking particulates were allowed to settle for a moment before taking aliquots for analysis.	Local Supermarket
Surface water	The sample material was allowed to reach room temperature and shaken thoroughly. After shaking particulates were allowed to settle for a moment before taking aliquots for analysis.	Osterdorfer Waterfall

Method Summary

Item	Activity
Principle of the Analytical Procedure	Homogenisation: shaken Extraction: acetonitrile shaking on a shaker Liquid-liquid Partition: citrate buffer salt tube, shaking, centrifugation Clean-up step: PSA SPE clean tube shaking, centrifugation Dilution: Acetonitrile/Water (50/50, v/v) Quantification: LC-MS/MS

Selectivity and Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 265→248 and m/z 265→182). Due to enhanced sensitivity mass transition m/z 265→248 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation. A reagent blank and two (2) control samples per matrix were extracted and analysed according to the method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions the samples showed no significant interference that would correspond to 30 % of LOQ at the retention time of the analyte in any investigated matrix therefore showing that the method is highly specific. Blank correction was not performed.

Matrix Effects

Matrix effect (%)	$= [(100 \cdot A_{\text{Matrix-Std}}) / (A_{\text{Solv-Std}})] - 100$
$A_{\text{Solv-Std}}$	Peak area of solvent standard
$A_{\text{Matrix-Std}}$	Peak area of matrix-matched standard

The matrix effects are summarised in the table below.

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Aclonifen (%)	
		Proposed for Quantification (m/z 265→248)	Proposed for Confirmation (m/z 265→182)
Drinking water	0.50 (10xLOQ)	(-) 16	(-) 13
Surface water	0.50 (10xLOQ)	(-) 4.5	(-) 0.1

Calibration

The linearity of the detector response was demonstrated by matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.015 ng/mL to 1.5 ng/mL. This range corresponds to a mass fraction level of 0.03 µg/L to 3.0 µg/L and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions and all matrices were linear since the regression residuals were randomly distributed on visual inspection. Furthermore, coefficients of determination (R^2) were ≥ 0.99 .

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was distributed over the whole analytical acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.10 µg/L was confirmed for aclonifen in drinking water and surface water. The LOD was defined as the lowest calibration standard, corresponding to 0.03 µg/L for drinking water and surface water, which is 30 % of the LOQ.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). Five (5) recovery determinations were performed at LOQ and at 10xLOQ, respectively. Analysis was performed by single extraction and single injection to the detection system.

Aclonifen							
Matrix	Fortification Level (µg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition <i>m/z</i> 265→248 (Proposed for Quantification)							
Drinking water	0.10	92.3, 92.1, 90.6, 94.0, 93.4	92.5	1.4	5	97.5	5.8
	1.0	106, 101, 99.5, 102, 104	103	2.4	5		
Surface water	0.10	103, 95.5, 95.9, 94.1, 98.3	97.4	3.7	5	96.1	3.4
	1.0	96.4, 92.6, 91.5, 96.5, 97.1	94.8	2.7	5		
Mass Transition <i>m/z</i> 265→182 (Proposed for Confirmation)							
Drinking water	0.10	88.5, 90.9, 93.7, 91.7, 94.0	91.8	2.4	5	98.0	6.9
	1.0	105, 106, 103, 102, 106	104	1.6	5		
Surface water	0.10	101, 105, 101, 101, 93.0	100	4.5	5	97.4	4.6
	1.0	97.0, 91.9, 93.3, 94.4, 96.6	94.6	2.3	5		

Stability of Standard Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection ($n = 5$).

Analyte	Solvent	Concentration (µg/mL)	Concentration after Dilution for Measurement (ng/mL)	Storage Period (Days)	Difference (in %) of stored stock solution compared to freshly prepared stock solution
Aclonifen (m/z 265→248)	Acetonitrile	1000	10	169	- 3.0

The mean peak area of the stock standard solution was within ± 10 % of the mean peak area of the freshly prepared solution indicating that stock solution is stable when stored at 1 °C to 10 °C in the dark for 169 days. Fortification and calibration solutions were prepared in the same solvent as stock solutions and thus did not require an extra testing.

Stability of Final Extracts

Following the first analysis, the final extracts of samples fortified at the 10xLOQ level together with one (1) control sample extract were stored at typically 1 °C to 10 °C in the dark. After storage the final extracts were analysed against freshly prepared calibration standards. One (1) mass transition was evaluated. The results obtained are summarised in the table below.

Matrix	Analyte	Fortification level (µg/L)	Days of storage *	Mean recovery Injection ($n = 5$) after Storage (%)	Rel. Std. Dev. ($n = 5$) (%)
Drinking water	Aclonifen (m/z 265→248)	1.0	9	103	3.6
Surface water	Aclonifen (m/z 265→248)	1.0	9	107	2.3

Conclusion

The method was successfully validated for the determination of aconitine in drinking water and surface water from the tested LOQ of 0.1 µg/L up to 1.0 µg/L according to the guidance document SAN-TE/2020/12830, rev. 2 for risk assessment and/or monitoring.

Reference List

- EN 15662:2018: "Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method".

- <https://mineralwasser-test.com/wasser/evian/>

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~~
New studies have been submitted.

Comments of zRMS:	The analytical method code: S24-105063 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830, rev. 2 The results of analytical method validation confirm that this method is suitable for determination of aconitine in Body Fluids (Human urine). The method is successfully validated and accepted.
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Reference:	KCP 5.3
Report	Development and Validation of an Analytical Method for the Determination of Aconitine in Body Fluids, Study Code: S24-105063, SENCIUC M.; 2025
GLP:	Yes
Duplication (if vertebrate study)	No
Acceptability:	Yes

Summary

Item	Activity, Result, Assessment
Study Scope	Validation of an analytical method according to guidance document SAN-TE/2020/12830, rev. 2 for risk assessment and/or monitoring
Analyte	Aconitine
Matrices	Human (urine)
Method Reference	Multi-residue method QuEChERS
LOQ	0.01 mg/L (lowest validated fortification level)

LOD	30 % of the LOQ (lowest calibration standard)								
Principle of the Analytical Procedure	Homogenisation: Shaken before taking aliquots for analysis Extraction: Addition of water and acetonitrile, shaking Liquid/Liquid Partition: Addition of magnesium sulphate, sodium chloride and sodium citrate, shake by hand, centrifugation Clean-up: Dispersive primary/secondary amine (PSA), shaker, centrifugation Dilution step: Factor 10 Sample concentration in final extract: 0.01 g sample per mL of extract Quantification: LC-MS/MS								
Selectivity and Specificity	Demonstrated by validation of two (2) mass transitions Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ.								
Matrix Effects on Analyte Detection	Insignificant (< 20 %)								
Calibration	Matrix-matched calibration standards A minimum of five (5) concentration levels Single determination Injection of standard solutions spread over the whole acquisition batch Concentration range: 0.03 ng/mL to 3.0 ng/mL Corresponding mass fraction range: 0.003 mg/L to 0.30 mg/L Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract The validated range does not exceed two (2) orders of magnitude								
Quantification	Linear regression with 1/x weighting Regression residuals randomly distributed Coefficients of determination (R ²) and correlation coefficients (R) ≥ 0.999								
Accuracy and Precision	Five (5) fortifications at 0.01 mg/L (LOQ) Five (5) fortifications at 0.1 mg/L (10x LOQ) Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2: <table><tr><td>Concentration Level (mg/kg / mg/L)</td><td>Range of Mean Recoveries (per level) (%)</td><td>Precision, Rel. Std. Dev. (per level) (%)</td></tr><tr><td>0.01 - 0.1</td><td>70 - 120</td><td>≤ 20</td></tr></table>			Concentration Level (mg/kg / mg/L)	Range of Mean Recoveries (per level) (%)	Precision, Rel. Std. Dev. (per level) (%)	0.01 - 0.1	70 - 120	≤ 20
Concentration Level (mg/kg / mg/L)	Range of Mean Recoveries (per level) (%)	Precision, Rel. Std. Dev. (per level) (%)							
0.01 - 0.1	70 - 120	≤ 20							
Stability of Analyte(s) in Standard Solutions	s Within ± 10 % for 79 days when prepared in acetonitrile and stored at typically 1 °C to 10 °C in the dark.								
Stability of Analyte in Sample Extracts	Recoveries within 70 % - 120 % in human (urine) extracts for 14 days when stored at typically 1 °C to 10 °C in the dark.								
Conclusion	The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.								

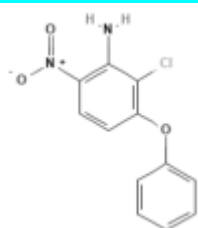
Materials and methods

Test/Reference items

Common name: Aclonifen

Chemical name: 2-chloro-6-nitro-3-phenoxybenzenamine

Chemical structure:



CAS number: 74070-46-5

Empirical formula: C₁₂H₉ClN₂O₃

Molecular weight: 264.66 g/mol

Supplier: Supelco; Purity analysed: 99.8 %
Date of certificate: 15 Jun 2022; Lot number BCCH7675
Date of receipt at Test Facility: May 2027
Storage conditions at Test Facility: Ambient (target)

Reagents and Materials

- Acetonitrile, HPLC grade (VWR, Art.No. 20060.320)
- LC-MS Formic acid, Promochem, LC-MS grade (FA)
- LC-MS Acetonitrile, Merck, LC-MS grade (ACN)
- LC-MS Water, Merck, LC-MS grade (H₂O)
- Dispersive SPE Citrate Extraction Tube (Supelco, Art.No. 55227-U)
- Dispersive SPE PSA SPE Clean-up Tube 1 (Supelco, Art.No. 55228-U)
- Milipore Water (supply at Test Facility)

Instrumentation and Apparatus

- Common laboratory glassware
- Analytical Balance, XS205DU (Mettler Toledo)
- Vortex mixer REAX top, Heidolph
- Horizontal shaker HS 260 B, IKA
- Ultrasonic bath Transonic 460, Elma Hans Schmidbauer
- Centrifuge Rotanta 460, Hettich

Matrix Type(s), Sample Origin, Preparation and Storage

Matrix Type(s)	Preparation	Origin
Human (urine)	The sample material was shaken before taking aliquots for analysis.	supplied by the Test Facility

Method Summary

Item	Activity, Result, Assessment
Principle of the Analytical Procedure	Homogenisation: Shaken before taking aliquots for analysis Extraction: Addition of water and acetonitrile, shaking Liquid/Liquid Partition: Addition of magnesium sulphate, sodium chloride and sodium citrate, shake by hand, centrifugation Clean-up: Dispersive SPE with primary/secondary amine (PSA), shaker, centrifugation Dilution step: Factor 10 Sample concentration in final extract: 0.01 g sample per mL of extract Quantification: LC-MS/MS

Selectivity and Specificity

LC-MS/MS determination was conducted with evaluation of two (2) mass transitions (m/z 265→248 and m/z 265→182). Due to enhanced sensitivity mass transition m/z 265→248 is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation. A reagent blank and two (2) control samples were extracted and analysed according to the

method to investigate the presence of residue and/or background interference at the retention time of the analyte. For both mass transitions, the samples showed no significant interference that would correspond to 30 % of LOQ at the retention time of the analyte therefore showing that the method is highly specific.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing peak areas of matrix-matched standards (90 % matrix amount) with solvent standards at comparable the same nominal concentrations.

Matrix effect (%)	$= [(100 \% \cdot A_{\text{Matrix-Std}} \cdot C_{\text{Solv-Std}}) / (A_{\text{Solv-Std}} \cdot C_{\text{Matrix-Std}})] - 100 \%$
$A_{\text{Solv-Std}}$	Peak area of solvent standard
$A_{\text{Matrix-Std}}$	Peak area of matrix-matched standard

Matrix / Commodity	Standard Concentration (ng/mL)	Matrix Effect for Aclonifen (%)	
		Proposed for Quantification (m/z 265→248)	Proposed for Confirmation (m/z 265→182)
Human (urine)	0.1 (LOQ)	(-) 4.2	(+) 1.8
	1.0 (10x LOQ)	(-) 3.5	(-) 4.5

Matrix suppression or enhancement was < 20 % for human (urine) for Aclonifen analyte and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the study.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.030 ng/mL to 3.0 ng/mL. This range corresponds to a mass fraction level of 0.003 mg/L to 0.30 mg/L and thus covers the range from no more than 30 % of the limit of quantification (LOQ) and at least + 20 % of the highest analyte concentration detected in any sample extract. The calibration curve does not exceed two (2) orders of magnitude. Linear regression was performed with 1/x-weighting. The calibration curves obtained for both ion mass transitions were linear since the regression residuals were randomly distributed. Furthermore, coefficients of determination (R^2) and correlation coefficients (R) were ≥ 0.999 .

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. The injection of standard solutions was distributed over the whole analytical acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ of the method is defined as the lowest analyte concentration at which the methodology was successfully validated. Thus, an LOQ of 0.01 mg/L was confirmed for aclonifen in human (urine). The LOD was defined as the lowest calibration standard, corresponding to 0.003 mg/L for human (urine), which is 30 % of the LOQ.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test / reference item and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). Five (5) recovery determinations were performed at LOQ and at 10x LOQ, respectively. Analysis was performed by single extraction and single

injection to the detection system.

Aclonifen							
Matrix	Fortification Level (mg/L)	Recovery (%)	Mean Recovery (%)	Rel. Std. Dev. (%)	Replicates	Overall Mean Recovery (%)	Overall Rel. Std. Dev. (%)
Mass Transition <i>m/z</i> 265→248 (Proposed for Quantification)							
Human (urine)	0.01	104, 97.3, 104, 101, 107	103	3.6	5	99.2	5.7
	0.1	86.4, 101, 98.5, 96.5, 96.9	95.9	5.8	5		
Mass Transition <i>m/z</i> 265→182 (Proposed for Confirmation)							
Human (urine)	0.01	103, 100, 104, 104, 114	105	4.9	5	101	5.8
	0.1	91.1, 97.8, 101, 103, 96.6	97.9	4.7	5		

All mean recovery values at fortification levels of 0.01 mg/L and 0.1 mg/L for two (2) mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2.

Concentration Level (mg/L)	Range of Mean Recoveries(per level) (%)	Precision, Rel. Std. Dev. (per level) (%)
0.01 - 0.1	70 - 120	≤ 20

All mean recovery values at fortification levels of 0.01 mg/L and 0.1 mg/L for two (2) mass transitions are within 70 % - 120 % with relative standard deviations ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2.

Stability of Standard Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5)

Analyte	Solvent	Concentration (µg/mL)	Concentration after Dilution for Measurement (ng/mL)	Storage Period (Days)	Difference (in %) of stored stock solution compared to freshly prepared stock solution
Aclonifen (<i>m/z</i> 265→248)	Acetonitrile	1000	10	79	- 1.8

The mean recovery of the stored solution was within ± 10 % of the mean recovery of the freshly prepared solution) indicating that stock solutions are stable when stored at 1 °C to 10 °C in the dark for 79 days.

Stability of Final Extracts

The final extracts of samples fortified at the 10xLOQ level together with one (1) control sample extract were stored at typically 1 °C to 10 °C in the dark. After storage the final extracts were analysed against freshly prepared calibration standards. One mass transition was reported. The results obtained are summarised in the table below.

Analyte in Matrix (Mass Transition)	Fortification level (mg/L)	Days of storage *	Mean recovery Injection (n = 5) after Storage (%)	Rel. Std. Dev. (n = 5) (%)
Aclonifen in Human (urine) (<i>m/z</i> 265→248)	0.10	14	93.3	1.8

The mean recovery values of the stored extracts were in the range of 70 % - 120 % with a relative standard deviation ≤ 20 % when analysed against freshly prepared calibration standards. Therefore, extracts are considered to be stable when stored at 1 °C to 10 °C for 14 days in the dark.

Conclusion

The method was successfully validated for the determination of aclonifen in/on human (urine) from the tested LOQ of 0.01 mg/L up to 0.1 mg/L according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring

Reference List

- EN 15662:2018: "Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE – Modular QuEChERS-method".

A 2.1.2.7 Other Studies/ Information (KCP 5.3)

Validation of method for determination of the content of aclonifen.

Comments of zRMS:	The analytical method code: ICB/106/2023 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANCO/3030/99 rev.5. The results of analytical method validation confirm that this method is suitable for determination of aclonifen and phenol as impurity. The method is successfully validated and accepted.
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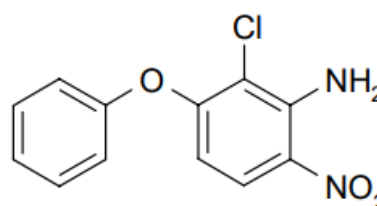
Reference:	KCP 5.2
Report	Validation of analytical method for CHR/H/AKO 600 SC for determination of aclonifen and phenol as impurity, Górka I., study code: ICB/106/2023
Deviations:	No
GLP:	Yes
Duplication (if vertebrate study)	No
Acceptability:	Yes

Validation was carried out according to Standard Operational Procedure SPB/179. Content of aclonifen at a level of 600 g/L in the test item was determined accordingly by liquid chromatography with diode array detection (HPLC-DAD).

Detected substance: Aclonifen

IUPAC Name: 2-chloro-6-nitro-3-phenoxyaniline
Molecular formula: $C_{12}H_9ClN_2O_3$
CAS No.: 74070-46-5
Purity: 97.0%
Series number: BCCH7675
Expiry date: May 2027
Manufacturing: Sigma Aldrich
Molecular weight: 264.7 g/mol

Structural formula:



Method validation

For validation method for determination of aclonifen were prepared two series of measurements of the test item, one without addition standard and second with addition standard. 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. To determine recovery, was prepared series of measurements (n=5) of the test item with the addition of standard. Calibration curve of active ingredient was prepared to determine the linear range. The average recovery and precision for validation level LOQ, was determined by prepared series of measurements (n=5) of solvent (acetonitrile) with standard addition. The average recovery and precision for validation level ULOQ, was determined by prepared series of measurements (n=5) of solvent (acetonitrile) with standard addition. Afterwards the sample was 4-fold diluted to fit the linear range of active ingredient. Specificity of the method was evaluated based on the analysis of sample chromatogram against chromatogram of standard aclonifen and peak purity.

Equipment and materials

- acetonitrile (Merck), - 85% phosphoric acid (VWR), - aclonifen standard; Sigma Aldrich; batch BCCH7675, prod. date: June 15, 2022; exp. date: May, 2027; storage conditions: 20±4°C, - standard stock solution of aclonifen in acetonitrile (for calibration), - working standard solutions of aclonifen in acetonitrile (for calibration), - standard stock solution of aclonifen in acetonitrile (for recovery), - analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16, - ultrasonic bath, W/29 (Branson, Taiwan), - liquid chromatograph with diode array detection (Shimadzu, Japan), WP/19, WP/42, - chromatography column type C18, 250 mm x 4,6 mm, 5 µm; (Zorbax Eclipse Plus, Agilent), K/11/HPLC, - chromatography column type C18, 100Å, 150 mm x 4,6 mm, 5 µm; (Luna Omega Polar, Phenomenex), K/15/HPLC, - chromatographic vials 1.5 mL with septa buthyl/Teflon, - volumetric flasks A class 10 mL, - measuring syringes 10 µL, 100 µL, 250 µL, 500 µL, 1000 µL.

Preparation of standard solutions

Comments of zRMS: Preparation of aclonifen standard stock solution in acetonitrile (for calibration).

Active ingredient	Weight [mg]	Flask volume [mL]	Concentration taking into account the purity of the standard [mg/mL]
aclonifen	10.0	10	0.998

Aclonifen standard solutions for calibration

Concentration of standard solution [mg/mL]	Volume of standard solution [µL]	Flask volume [mL]	Concentration of working standard solutions [µg/mL]	Calibration level
0.998 (aclonifen)	10	10	0.998	Cal 1
0.998 (aclonifen)	100	10	9.98	Cal 2
0.998 (aclonifen)	250	10	24.95	Cal 3
0.998 (aclonifen)	500	10	49.9	Cal 4
0.998 (aclonifen)	1000	10	99.8	Cal 5

Aclonifen standard solution for determination recovery

Active ingredient	Weight [mg]	Flask volume [mL]	Concentration taking into account the purity of the standard [mg/mL]
Aclonifen	49.0	10	4.89

Chromatography parameters

Parameters	Primary chromatographic system	Secondary chromatographic system
Column	K/11/HPLC	K/15/HPLC
Analysis time	10 min	10 min
Flow	1.4 mL/min	1.4 mL/min
Column temperature	25°C	25°C
Injection	20 µL	20 µL
Detector DAD	237 nm	237 nm
Mobile phase	A–Acetonitrile, D–0.1% H ₃ PO ₄	A–Acetonitrile, D–0.1% H ₃ PO ₄
Isocratic program	phase A - 80%, D – 20%	phase A-70%, phase D–30%

Criteria of method acceptance

According to SPB/179, the parameters obtained as a result of validation should meet the following criteria:

- linearity $R^2 \geq 0.99$;
- specificity;
- precision for aclonifen ≤ 1.49 (validation level 100%);
- standard addition at the level of 20-30% of the content in test item;
- recovery for aclonifen 97-103 (validation level 100%);
- precision for aclonifen ≤ 2.65 (validation level LOQ);
- recovery for aclonifen 90-110 (validation level LOQ);
- precision for aclonifen ≤ 1.39 (validation level ULOQ);
- recovery for aclonifen 97-103 (validation level ULOQ);
- Horwitz ratio ≤ 1.0

Calculations and results

a) Linearity

In order to check the linearity of aclonifen, calibration curve was prepared using standard solutions. A graph of the peak area to the concentration of aclonifen was plotted. The resulting curve is linear in the tested concentrations.

Linearity range of aclonifen is from 1.076 to 312.96 µg/mL.

Correlation coefficient R^2 is 0.9999991

Linear regression is described by equation: $f(x) = 1.83502 \cdot 10^{-5} - 0.0435281$ for primary chromatographic system.

Correlation coefficient R^2 is 0.9999926

Linear regression is described by equation: $f(x) = 1.85925 \cdot 10^{-5} - 0.152390$ for secondary chromatographic system.

b) Specificity

Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard aclonifen and peak purity. Analysis showed no overlapping of determined ingredi-

ent signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.

c) Determination of the average content of aclonifen and precision

Final result was calculated according to following formula:

$$X = \frac{C \times V}{N} \times 0.1$$

where:

X – concentration of aclonifen [%],

C – result from the chromatogram [µg/mL]

V – the volume to which the test item was diluted [mL]

N – weight of analysed test item [mg].

Average content of aclonifen from both systems is **49.96%**.

Precision was calculated according to following equations:

$$RSD [\%] = \frac{SD}{\bar{C}_U} \times 100\%$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (C_{U_i} - \bar{C}_U)^2}{n - 1}}$$

$$\bar{C}_U = \frac{\sum_{i=1}^n C_{U_i}}{n}$$

where:

C_{U_i} – concentration of active ingredient [µg/mL]

n – amount of measurements.

Results of precision and Horwitz ratio at 100% validation level for aclonifen

Chromatographic system	Active ingredient	Precision [%]	Horwitz ratio
Primary	Aclonifen	0.73	0.49
Secondary	Aclonifen	0.39	0.26

Obtained results meet the criteria.

d) Determination recovery for aclonifen

Recovery was calculated according to following equation

$$Rec[\%] = \frac{C_F - \bar{C}_U}{C_A} \times 100\%$$

where:

C_F – concentration of active ingredient in the test item in the sample with standard addition [$\mu\text{g/mL}$]

$\overline{C_U}$ – average concentration of active ingredient [$\mu\text{g/mL}$]

C_A – standard addition [$\mu\text{g/mL}$]

Results of recovery for aclonifen from both systems

Chromatographic system	Active ingredient	Recovery [%]	Standard addition
Primary	Aclonifen	97.73-102.13	23.53
Secondary	Aclonifen	97.76 – 102.03	23.45

Obtained results meet the criteria

e) Determination of average recovery and precision for validation level LOQ for aclonifen

Recovery for validation level LOQ for aclonifen was calculated according to following equation:

$$Rec[\%] = \frac{C_F}{C_A} \times 100\%$$

Where:

C_F – results from the chromatogram of solvent (acetonitrile) with standard addition [$\mu\text{g/mL}$]

C_A – standard addition [$\mu\text{g/mL}$]

Precision was calculated according to following equations:

$$RSD [\%] = \frac{SD}{\overline{C_U}} * 100\%$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (C_{U_i} - \overline{C_U})^2}{n - 1}}$$

$$\overline{C_U} = \frac{\sum_{i=1}^n C_{U_i}}{n}$$

Where:

C_{v_i} – results from the chromatogram [$\mu\text{g/mL}$]

n – amount of measurements

Results of average recovery, precision and Horwitz ratio for aclonifen for validation level LOQ from both chromatographic system

Chromatographic system	Active ingredient	Precision [%]	Horwitz ratio	Average recovery [%]
Primary	Aclonifen	0.49	0.18	101.1
Secondary	Aclonifen	0.97	0.37	92.0

Obtained results meet the criteria

f) Determination of average recovery and precision for validation level ULOQ for aclonifen

Recovery for validation level ULOQ for aclonifen was calculated according to following equation:

$$Rec[\%] = \frac{C_F}{C_A} \times 100\%$$

Where:

C_F – results from the chromatogram of solvent (acetonitrile) with standard addition multiply by dilution factor [$\mu\text{g/mL}$]

C_A – standard addition [$\mu\text{g/mL}$]

Precision was calculated according to following equations:

$$RSD [\%] = \frac{SD}{\bar{C}_U} * 100\%$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (C_{U_i} - \bar{C}_U)^2}{n - 1}}$$

$$\bar{C}_U = \frac{\sum_{i=1}^n C_{U_i}}{n}$$

Where:

C_{U_i} – results from the chromatogram [$\mu\text{g/mL}$]

n – amount of measurements

Chromatographic system	Active ingredient	Precision [%]	Horwitz ratio	Average recovery [%]
Primary	Aclonifen	0.58	0.42	100.0
Secondary	Aclonifen	0.51	0.37	100.2

Obtained results meet the criteria

Comments of zRMS:	The analytical method code: ICB/106/2023 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANCO/3030/99 rev.5. The results of analytical method validation confirm that this method is suitable for determination of aclonifen and phenol as impurity. The method is successfully validated and accepted.
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Validation of method for determination of the content of phenol as impurity.

Reference: KCP 5.2

Report	Validation of analytical method for CHR/H/AKO 600 SC for determination of aclonifen and phenol as impurity, Górka I., study code: ICB/106/2023
Deviations:	No
GLP:	Yes
Duplication (if vertebrate study)	No
Acceptability:	Yes

Validation was carried out according to Standard Operational Procedure SPB/179. Content of phenol in the test item was determined by liquid chromatography with diode array detection (HPLC-DAD).

Detected substance: ~~Aclonifen~~ Phenol

IUPAC Name: Phenol (Benzenol)

Molecular formula: C_6H_5OH

CAS No.: 108-95-2

Maximum content of impurity: 5 g/kg

Purity: $\geq 98.0\%$

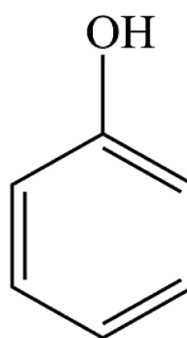
Series number: ~~BCCH7675~~ BCCG0109

Expiry date: ~~May 2027~~ APR 2026

Manufacturing: Sigma Aldrich

Molecular weight: 94.113 g/mol

Structural formula:



Method validation

For validation method for determination of phenol in the formulation SC which contains aclonifen as active ingredient were prepared two series of measurements of the test item, one without addition standard and second with addition standard. To determine the average content of phenol (validation level 100%) and precision, was prepared series of measurements ($n=5$) of the test item without standard addition. To determine recovery, was prepared series of measurements ($n=5$) of the test item with the addition of standard. Calibration curve of phenol was prepared to determine the linear range. The average recovery and precision for validation level LOQ, was determined by prepared series of measurements ($n=5$) of solvent (acetonitrile) with standard addition. Specificity of the method was evaluated based on the analysis of sample chromatogram against chromatogram of standard phenol and peak purity.

Equipment and materials

- acetonitrile (Merck), - 85% phosphoric acid (VWR), - phenol standard; batch BCCG0109, prod. date: August 17, 2021; exp. date: April, 2026; storage conditions: $4\pm 2^\circ C$, - phenol standard stock solution in acetonitrile, - working standard solution of phenol in acetonitrile (for calibration), - working standard solutions of phenol in acetonitrile (for calibration), - working standard solution of phenol in acetonitrile (for recovery), - analytical balance – accuracy 0.0001 g (Ohaus, Switzerland), WP/16, - ultrasonic bath, W/29 (Branson, Taiwan), - syringe filters with a pore diameter of 0.45 μm , - liquid chromatographs with diode array detection (Shimadzu, Japan), WP/19, WP/42, - chromatography column type C18, 100Å, 250 mm x 4,6 mm, 5 μm (Luna, Phenomenex), K/17/HPLC, - chromatography column type C18, 100Å, 250 mm x 4,6 mm, 5 μm ; (Luna, Phenomenex), K/1/HPLC, - chromatographic vials 1,5 mL with septa buthyl/teflon, - volumetric flasks class A, 10 mL, - measuring syringes 10 μL , 50 μL , 500 μl and 1000 μL .

Preparation of standard solutions

Preparation of phenol standard stock solution in acetonitrile

Impurity	Concentration [mg/mL]	Flask volume [mL]	Concentration taking into account the purity of the standard [$\mu\text{g/mL}$ mg/mL]
Phenol	10.2	10	10.2 1.02

Phenol working standard solution in acetonitrile (for calibration)

Impurity	Concentration [mg/mL]	Volume [μL]	Flask volume [mL]	Concentration taking into account the purity of the standard [$\mu\text{g/mL}$]
Phenol	1.02	1000	10	102.0

Phenol working standard solutions for calibration in acetonitrile (for calibration)

Concentration of standard solution [mg/mL]	Volume of standard solution [μL]	Flask volume [mL]	Concentration of working standard solutions [$\mu\text{g/mL}$]	Calibration level
102.0	10	10	0.102	Cal 1
102.0	50	10	0.510	Cal 2
102.0	500	10	5.100	Cal 3
102.0	1500	10	15.300	Cal 4
102.0	2000	10	20.400	Cal 5

Phenol working standard solution in acetonitrile (for recovery)

Impurity	Concentration [mg/mL]	Volume [μL]	Flask volume [mL]	Concentration taking into account the purity of the standard [$\mu\text{g/mL}$]
Aclonifen	1.02	100	10	10.2

Chromatography parameters

Parameters	Primary chromatographic system	Secondary chromatographic system
Column	K/17/HPLC	K/1/HPLC

Analysis time	45 min	45 min
Flow	1.4 mL/min	1.2 mL/min
Colum temperature	25 °C	25 °C
Injection	30 µL	30 µL
Detector DAD	270 nm	270 nm
Mobile phase	A–Acetonitrile, D–0.1% H ₃ PO ₄	A–Acetonitrile, D– 0.1% H ₃ PO ₄
Gradient program	10 min phase A - 25%, phase D – 75% 15 min phase A - 100%, phase D – 0% 20 min phase A - 100%, phase D – 0% 25 min phase A - 100%, phase D – 0% 35 min phase A - 25%, phase D – 75% 45 min phase A - 25%, phase D – 75%	7 min phase A - 25%, phase D – 75% 15 min phase A - 100%, phase D – 0% 20 min phase A - 100%, phase D – 0% 25 min phase A - 100%, phase D – 0% 35 min phase A - 25%, phase D – 75% 45 min phase A - 25%, phase D – 75%

Criteria of method acceptance

According to SPB/179, the parameters obtained as a result of validation should meet the following criteria:

- linearity $R^2 \geq 0.99$;
- specificity;
- precision for phenol ≤ 7.48 (validation level 100%);
- precision for phenol ≤ 8.15 (validation level LOQ);
- recovery for phenol 70-130% (validation level 100%);
- recovery for phenol 70-130% (validation level LOQ);
- Horwitz ratio ≤ 1.0

Calculations and results

a) Linearity

In order to check the linearity of phenol, calibration curve was prepared using standard solutions. A graph of the peak area to the concentration of phenol was plotted. The resulting curve is linear in the tested concentrations.

Linearity range of phenol is from 0.184 to 20.400 µg/mL.

Correlation coefficient R^2 is 0.9999731

Linear regression is described by equation: $f(x) = 5.36705 \cdot 10^{-5} - 0.0202411$ for primary chromatographic system.

Correlation coefficient R^2 is 0.9999527

Linear regression is described by equation: $f(x) = 4.44378 \cdot 10^{-5} - 0.0188189$ for secondary chromatographic system.

b) Specificity

Specificity of the method was evaluated based on the analysis of chromatogram for sample against chromatogram of standard phenol and peak purity. Analysis showed no overlapping of determined impurity signal with the signals of matrix components under method conditions, hence method specificity criterion is fulfilled.

c) Determination of the average content of phenol

Final result was calculated according to following formula:

$$X = \frac{C \times V}{N} \times 0.1$$

Where:

X – concentration of phenol [%],
 C – result from the chromatogram [$\mu\text{g/mL}$]
 V – the volume to which the test item was diluted [mL]
 N – weight of analysed test item [mg].

Average content of phenol from both systems is **0.00109%**

Precision was calculated according to following equations:

$$RSD [\%] = \frac{SD}{\bar{C}_U} * 100\%$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (C_{U_i} - \bar{C}_U)^2}{n - 1}}$$

$$\bar{C}_U = \frac{\sum_{i=1}^n C_{U_i}}{n}$$

Where:

C_{U_i} – concentration of phenol [$\mu\text{g/mL}$]
 n – amount of measurements.

Results of precision and Horwitz ratio at 100% validation level for phenol

Chromatographic system	Impurity	Precision [%]	Horwitz ratio
Primary	Phenol	3.92	0.52
Secondary	Phenol	1.83	0.24

d) Determination recovery for phenol

Recovery was calculated according to following equation:

$$Rec[\%] = \frac{C_F - \bar{C}_U}{C_A} \times 100\%$$

Where:

C_F – concentration of phenol in the test item in the sample with standard addition [$\mu\text{g/mL}$]
 $\bar{C}_U$ – average concentration of phenol [$\mu\text{g/mL}$]
 C_A – standard addition [$\mu\text{g/mL}$]

Results of recovery for phenol from both systems

Chromatographic system	Impurity	Recovery [%]	Standard addition [%]
Primary	Phenol	76.63 – 123.70	25.53
Secondary	Phenol	85.26 – 106.05	24.94

Obtained results meet the criteria

e) Determination of average recovery and precision for validation level LOQ for phenol

Recovery for validation level LOQ for phenol was calculated according to following equation:

$$Rec[\%] = \frac{C_F}{C_A} \times 100\%$$

Where:

C_F – results from the chromatogram of solvent (acetonitrile) with standard addition [$\mu\text{g/mL}$]

C_A – standard addition [$\mu\text{g/mL}$]

Precision was calculated according to following equations:

$$RSD [\%] = \frac{\bar{SD}}{\bar{C}_U} * 100\%$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (C_{U_i} - \bar{C}_U)^2}{n - 1}}$$

$$\bar{C}_U = \frac{\sum_{i=1}^n C_{U_i}}{n}$$

Where:

C_{v_i} – results from the chromatogram [$\mu\text{g/mL}$]

n – amount of measurements

Results of average recovery, precision and Horwitz ratio for phenol for validation level LOQ from both chromatographic systems

Chromatographic system	Impurity	Precision [%]	Horwitz ratio	Average recovery [%]
Primary	Phenol	7.35	0.90	103.8
Secondary	Phenol	6.09	0.75	103.5

Obtained results meet the criteria

Comments of zRMS:	The analytical method code: VAL/24/2023 was fully validated in term of specificity, linearity, repeatability, accuracy according to SANTE/2020/12830 rev. 2. To compensate matrix effect, there was used matrix-matched calibrations. The results of analytical method validation confirm that this method is suitable for the determination of residues of aclonifen in potato.
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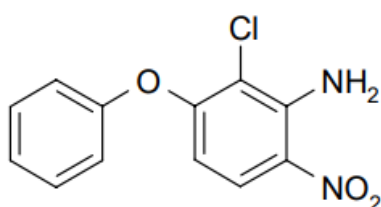
Validation of an analytical method for the determination of residues of aclonifen in potato.

Reference:	KCP 7.1
Report	Validation of an analytical method for the determination of residues of aclonifen in potato, study director: Głowiak K.; Validation Study No.: VAL/24/2023
Guidance:	SANTE/2020/12830 rev. 2 14 February 2023
Deviations:	No
GLP:	Yes
Acceptability:	Yes
Duplication (if vertebrate study)	No

Aclonifen

IUPAC Name: 2-chloro-6-nitro-3-phenoxyaniline
Molecular formula: $C_{12}H_9ClN_2O_3$
CAS No.: 74070-46-5
Purity: 98.03%
Lot: G1418464
Expiry date: 10.01.2028
Molecular weight: 264.66 g/mol
Storage: $20^{\circ}C \pm 4^{\circ}C$

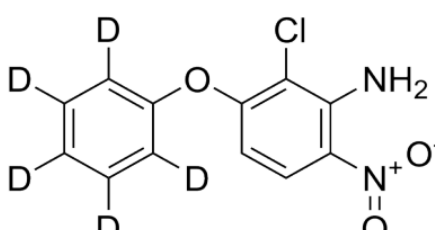
Structural formula:



Aclonifen D5 (phenyl D5)

IUPAC Name: 2-chloro-6-nitro-3-phenoxyaniline-d5
Molecular formula: $C_{12}D_5H_4ClN_2O_3$
CAS No.: 2469279-06-7
Purity: 96.9%
Lot: 1347322
Expiry date: 22.02.2027
Molecular weight: 269.70 g/mol
Storage: $20^{\circ}C \pm 4^{\circ}C$

Structural formula:



Method Validation

The purpose of this study was to validate an analytical method for the determination of residues of aclonifen in potato. Specimen extraction and determination of residues was performed using QuEChERS technique. The specimens were prepared, extracted and analyzed following analytical procedures:

- ANALYTICAL PROCEDURE DPL-67 Determination of residues of aclonifen in potato using QuEChERS method and LC-MS/MS tandem mass spectrometry, based on: EN 15662:2018 Foods of plant origin. Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE. Modular QuEChERS-method

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

The limit of detection (LOD) in potato that was expressed as the lowest calibration standard was 0.001 mg/kg, 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 0.010 mg/kg for aclonifen in potato.

Equipment and materials

- Electronic balance Class I; WP-011/W/S/LF • Electronic balance Class II; WP-012/W/S/LF, WP-013/W/S/LF • Freezers - storage of: - analytical standards; W-082/S/LF - analytical samples before the analytical part; W-093/LF - analytical samples extracts—freezing during extraction/preparation of samples; W-084/S/LF - analytical samples extracts until the end of the instrumental analysis; W-084/S/LF • Centrifuge (> 3000 rpm); W-076/LF • Cutter (knife grinder); W-133/LF, W-096/LF • Polypropylene centrifuge tubes, with screw caps, 25 and 50 mL • Plastic cups (stackable), 100 mL, for the storage of archived samples • Volumetric flasks, 5 and 10 mL (class A) • Automatic pipettes: 10–200 µL (WP-111/S/LF); 0.5-5.00 mL (WP-102/S/LF); 1.00-10.0 mL (WP103/S/LF); 50-1000 µL (WP-138/S/LF); • Vials with screw caps, amber glass, 10 mL • Injection vials, 2.5 mL, amber glass, screw caps with septa butyl/PTFE • Syringe filters RC 0.22 µm, 25 mm • Liquid Chromatography Instrument LC-MS/MS Sciex Qtrap 6500+ with SeleXION+ ; WP-144/K/S/ko/LF • Chromatographic column: Synergi™ 4 µm Fusion-RP 80 Å, 100 x 2 mm, S/N H22-295772 (Phenomenex)

- Water for LC-MS, Lot: Z0911433406 • Acetonitrile, CH₃CN, hypergrade for LC-MS, Lot: I1316029348 • Methanol, CH₃OH, HPLC grade, Lot: I1315435402 • Acetone, C₃H₆O, Lot: 211007A002 • Ammonium acetate for LC-MS, Lot: AM1889304 • Buffer salt mixture, Lot: 1124, containing: - 4g of anhydrous magnesium sulfate, MgSO₄ - 1g of sodium chloride, NaCl - 1g of trisodium citrate dihydrate, C₅H₅Na₃O₇·2H₂O - 0.50g of disodium hydrogencitrate sesquihydrate, C₆H₆Na₂O₇·1,5H₂O

Analysis

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

For each analyte, two mass transitions were evaluated and used for quantification. Representative chromatograms are shown in this report. A third mass transition was monitored for confirmation of peak iden-

tity, but was not used for quantification.

Compound name	Polarization (+)	
	M/Z (Quantifer)	M/Z (Qualifier)
Aclonifen	265.00 → 182.00 265.00 → 218.00	265.00 → 194.00
Aclonifen D5	270.00 → 187.00	270.00 → 223.00 270.00 → 253.00

Instrument settings:

Liquid Chromatograph LC-MS/MS Sciex Qtrap 6500+ with SelexION+ (WP-144/K/S/ko/LF) consists of:

Degazer DGU-405

Two pumps LC-40D XR

Autosampler SIL-40C XR

Column oven CTO-40C

Compressor, generator PEAK Genius 1024

HPLC Column – Synergi™ 4 µm Fusion-RP 80 Å, 100 x 2 mm, S/N H22-295772 (Phenomenex)

Calculations and results

a) Specificity and selectivity

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

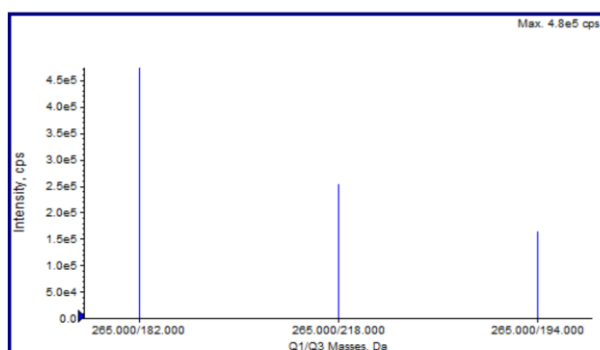


Figure 1. Mass spectrum of aclonifen

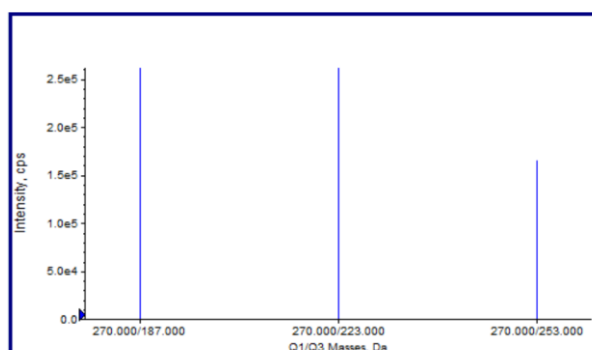


Figure 2. Mass spectrum of aclonifen D5

b) Linearity

The linearity of the detector response was demonstrated by single determination of calibration standards at concentration levels ranging from 1 ppb to 500 ppb. 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 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 concentra-

tion.

c) Limit of quantification/detection

The LOQ is the lowest validated fortification level for which an average recovery in the range of 70 – 120% (60 – 120 % in case of level ≤ 0.01 mg/kg) and $RSD \leq 20$ % (≤ 30 % in case of level ≤ 0.01 mg/kg) is achieved. Limit of detection (LOD) was established at 0.001 mg/kg. The limit of detection (LOD) was expressed as the lowest calibration standard. LOQ was successfully established at 0.010 mg/kg for aclonifen in potato

d) Precision, accuracy and uncertainty

Recovery data was generated from five samples fortified at the limit of quantification (LOQ) and five samples fortified at the 10-fold higher concentration than the LOQ (10 x LOQ). Precision of the method was determined as the relative standard deviation (RSD) of recovery at each fortification level. The mean recovery at fortification level of 0.01 mg/kg (LOQ) should be in the range of 60 – 120% with $RSD \leq 30$ %, and recovery at fortification level of 0.1 mg/kg (10xLOQ) should be in the range of 70 – 120% with $RSD \leq 20$ %

The recovery data was calculated according to equation:

$$R = \frac{C_R \cdot 100}{C_F}$$

Where:

R - recovery [%]

C_R - analyzed concentration of analyte in the fortified specimen [mg/kg]

C_F - nominal concentration of analyte in the fortified specimen [mg/kg]

Relative Standard Deviation (RSD %) was calculated according to equation:

$$RSD = \frac{s}{\bar{x}}$$

where:

s – standard deviation obtained from a series of results

$\bar{x}$ – average received from the result series

Additionally Expanded Uncertainty was calculated using the within-laboratory reproducibility RSD combined with estimates of the method and the laboratory bias.

Expanded Uncertainty (U_c %) was calculated according to equation:

$$U_c = 2 \cdot \sqrt{(RSD)^2 + (100 - Rec)^2}$$

Where:

U_c – Expanded Uncertainty

RSD – Relative Standard Deviation, expressed as a measure of repetition (precision)

Rec – accuracy of the method, expressed in the recovery of method

e) Matrix effects

Matrix effects, expressed in % enhancement or suppression can be evaluated according to the following

equation:

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

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

In case of this validation study, calibration standard at 10 ng/mL in solvent was additionally analyzed and matrix effects were calculated according to the equation above.

Matrix effect [%] = **-31.78**

Comments of zRMS:	In accordance with the provisions in SANTE/2020/12830 Rev.2, 24 February 2023, matrix effects are considered significant if they exceed $\pm 20\%$. In the study the matrix effect exceeded the $\pm 20\%$ and reached - 31.78%. According to the study report: <i>To compensate matrix effect, there was used matrix-matched calibrations in all cases.</i> <i>Figures of matrix blank samples illustrate the effect of the blank matrix for the determination of aclonifen. In the table and on chromatograms showed below, I present the details (peak areas) of blank and fortified samples analysis at the LOQ level (0.010 mg/kg). Based on the background verification of the chromatograms and possible interference at the retention time of the peak of the determined substance, it was found that all matrices meet the <30% LOQ criterion, however "matrixmatched" calibration was used in all cases.</i>
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TRANSITION 265.0→182.0

Chromatogram of calibration blank showing a single peak at 8.069 minutes. The y-axis is Intensity (0.0e0 to 4.5e5) and the x-axis is Time, min (7.5 to 9.0). The legend indicates: cal blank - aclonifen 1 265.0 / 182.0, aclonifen 2 (265.0 / 218.0), and aclonifen 3 (265.0 / 194.0).

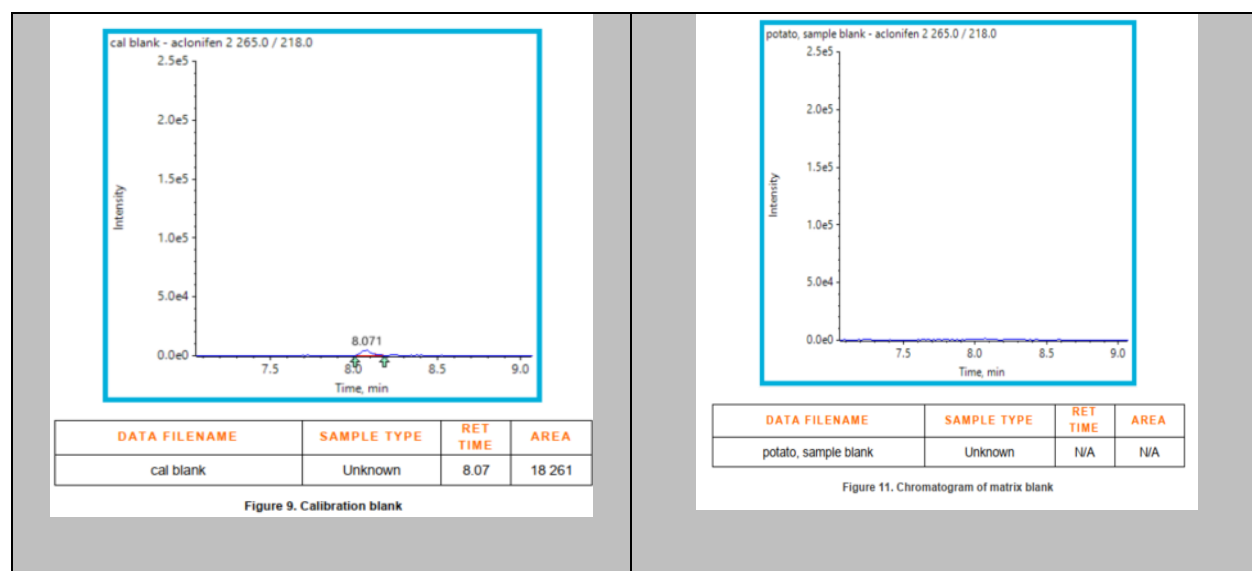
DATA FILENAME	SAMPLE TYPE	RET TIME	AREA
cal blank	Unknown	8.07	35 286

Figure 3. Calibration blank

Chromatogram of matrix blank (potato, sample blank) showing no significant peaks. The y-axis is Intensity (0.0e0 to 4.5e5) and the x-axis is Time, min (7.5 to 9.0). The legend indicates: potato, sample blank - aclonifen 1 265.0 / 182.0, aclonifen 2 (265.0 / 218.0), and aclonifen 3 (265.0 / 194.0).

DATA FILENAME	SAMPLE TYPE	RET TIME	AREA
potato, sample blank	Unknown	N/A	N/A

Figure 5. Chromatogram of matrix blank



Summary

Parameter	Criterion of acceptance	Results obtained for Aclonifen (transition 265.00 → 182.00)	Results obtained for Aclonifen (transition 265.00 → 218.00)
Specificity/selectivity	fulfilled		
Linearity	$R^2 \geq 0.99$	a) $R^2 = 0.9998$	b) $R^2 = 0.9999$
Quantification (target) ion	-	265.00→182.00	265.00→218.00
Qualification (ref) Ion(s)	-	265.00→218.00 265.00→194.00	-
Limit of quantification (LOQ)	-	0.010 mg/kg	0.010 mg/kg
Limit of detection (LOD)	-	0.001 mg/kg	0.001 mg/kg

a) Transition 265.00 → 182.00

TRANSITION 265.00→182.00

Calibration for aclonifen 1: $y = 1.77509x + 2.52461e-4$ ($r = 0.99991$, $r^2 = 0.99982$) (weighting: $1/x$)

b) Transition 265.00 → 218.00

TRANSITION 265.00 → 218.00

Calibration for aclonifen 2: $y = 0.97502 x + -5.94548e-4$ ($r = 0.99997$, $r^2 = 0.99993$) (weighting: 1 / x)

Precision and accuracy

Transition: 265.00 → 182.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.010	0.010	100.9	0.10	0.10	102.1
	0.0098	98.2		0.10	102.3
	0.010	101.1		0.10	101.1
	0.0099	99.1		0.10	104.1
	0.010	101.6		0.098	98.4
Avarage	0.010	100.2	Avarage	0.10	101.6
SD	0.00015	1.46	SD	0.0021	2.09
RSD [%]	1.46		RSD [%]	2.06	
Uncertainty [%]	2.9		Uncertainty [%]	5.2	

Transition: 265.00 → 218.00

Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]	Fortification level [mg/kg]	Obtained result [mg/kg]	Recovery [%]
0.010	0.0099	98.8	0.10	0.10	102.5
	0.0096	95.8		0.099	98.5
	0.0097	96.9		0.099	99.1
	0.0096	96.4		0.10	99.7
	0.0097	97.3		0.099	98.9
Avarage	0.0097	97.0	Avarage	0.10	99.7
SD	0.00011	1.11	SD	0.0016	1.63
RSD [%]	1.14		RSD [%]	1.63	
Uncertainty [%]	6.3		Uncertainty [%]	3.3	