FINAL REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: H-01-2022

Product name(s): Terbutylazyna 500 SC

Chemical active substance:

terbuthylazine, 500 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorization)

Applicant: ProAgri International Sp. z o.o.

Submission date: April 2024

MS Finalisation date: 11.2024**;** 03.2025

Version history

When	What
November 2024	ZRMs evaluated dRR submitted by Applicant
March 2025	The final Registration Report

Table of Contents

5 Analytical methods

5.1 Conclusion and summary of assessment

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

Sufficiently sensitive and selective analytical methods are available for determination of the active substances and relevant impurities in the plant protection product Terbutylazyna 500 SC. No data gaps.
Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.

Commodity/crop	Supported/ Not supported
Maize (seeds)	Supported

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

5.2.1 Analysis of the plant protection product (KCP 5.1.1)

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

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

Comments of Evaluator:	The analytical method (HPLC-DAD) for determination of active substance Terbuthylazine in the formulation Terbutylazyna 500 SC has been submitted and meets criteria of specificity, linearity and precision according to the requirements SANCO 3030/99 rev 5, therefore the method is acceptable.

Reference:	5.1.1/01
Report	Analytical Method Validation for Active Ingredient and impurities Content Determination of the H-01-2022 product in Order to Provide an Analytical Certificate, Condorelli A.M.M., 2023, report no. 22363-01C
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study objective is the analytical method validation for active ingredient and impurities content Determination of the H-01-2022 product in Order to fulfil to the requirements of Regulation (EC) No

1107/2009 and commission implementing Regulation (EU) No 2021/824. A HPLC-DAD method was validated for determination of terbuthylazine and its relevant impurities in H-01-2022 product. Details are described in Appendix 2, point A 2.1.1.1.

Test item

Name	H-01-2022
Active substance	Terbuthylazine
IUPAC name of a.s.	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine
CAS Number of a.s.	5915-41-3
Nominal content:	500 g terbuthylazine/L
Rate of Use (Max)	1.5 L/ha (750 g terbuthylazine/ha)
Rate of Use (Min)	1.0 L/ha (500 g terbuthylazine/ha)
Physical state	Liquid
Formulation type	Suspension Concentrate (SC)
Batch number	1/23
Manifacturing date	January 2023
Expiry date	January 2025
Storage conditions	Ambient temperature

Equipment and apparatus

- Standard laboratory glassware/equipment
- Analytical Balance, OHAUS, PX224 Pioneer
- Technical balance accurate to 0.01 g, Sartorius, BP 3100 P
- HPLC equipped with DAD detector, Agilent, 1290 Infinity II
- LC with TRIPLE TOF 4600 AB SCIEX detector, Agilent ABSCIEX, HPLC Series 1290 TRIPLE TOF 4600
- Chromatographic column 250*4.6 mm *5µm particle size, Thermoscientific, Hypersil ODS
- Single use syringe with PTFE filter 0.45 µm pre size, Scharlab, PTFE
- Ultrasonic bath, AGE, Uniset AC
- Hydrometer (Series)

Instrument conditions:

LC system	UHPLC Series 1290 INFINITY II Agilent		
Analytical column	Thermoscientific, Hypersil ODS, C18 5 µm 250x4.6 mm		
Solvent A:	Water		
Solvent B:	Acetonitrile		
Flow:	1 mL/min		
Pump Parameters	Time [min]	A (%)	B (%)
	0.00	70	30
	10.0	45	55
	14.0	45	55
	21.0	20	80
	21.01	5	95
	24.0	5	95
	24.01	70	30
	28.0	70	30
Oven temperature :	30 °C		
Injection volume:	5 µL		
Detector wavelength:	220 nm		
Chromatogram time:	28 min		
Retention times:	~10.8 min (Simazine) ~13.6 min (Atrazine) ~17.4 min (Propazine) ~18.4 min (Terbuthylazine)		

Validation - Results and discussions

Table 5.2-1: Methods suitable for the determination of active substances terbuthylazine in plant protection product H-01-2022

	Terbuthylazine
Author(s), year	Condorelli A.M.M., 2023
Principle of method	HPLC-DAD
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of the analytical method was assessed using five standard solutions in the concentration range: 0.0428-0.1499 mg/mL (21.4-75.0 %w/w). y=83817.1929x-62.7218 Correlation coefficient: r^2 = 0.9993 Acceptability: $r^2 \geq 0.98$
Precision – Repeatability Mean n = 5 (%RSD)	44.8 % w/w RSD % = 0.49 Horowitz %: 1.51 Hr = 0.33 Acceptability: Hr ≤ 1
Accuracy n = 2 (% Total Recovery)	~44.8 % w/w 101.5% Acceptability: 97% - 103%
Interference/ Specificity	No interference. Acceptability: Interference <3% of analyte
Comment	No comments.

Conclusion

A HPLC-DAD method was validated for determination of terbuthylazine. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

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

Comments of Evaluator:	The analytical method (HPLC-DAD) for determination of relevant impurities in the formulation Terbutylazyna 500 SC has been submitted and meets criteria of specificity, linearity and precision according to the requirements SANCO 3030/99 rev 5, therefore the method is acceptable

Reference:	5.1.1/02
Report	Analytical Method Validation for Active Ingredient and impurities Content Determination of the H-01-2022 product in Order to Provide an Analytical Certificate, Condorelli A.M.M., 2023, report no. 22363-01C
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study objective is the Analytical Method Validation for Active Ingredient and impurities content Determination of the H-01-2022 product in Order to fulfil to the requirements of Regulation (EC) No 1107/2009 and commission implementing Regulation (EU) No 2021/824. A HPLC-DAD method was validated for determination of terbuthylazine and its relevant impurities in H-01-2022 product. Details are described in Appendix 2, point A 2.1.1.1.

Test item

Name	H-01-2022
Active substance	Terbuthylazine
IUPAC name of a.s.	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine
CAS Number of a.s.	5915-41-3
Nominal content:	500 g terbuthylazine/L
Rate of Use (Max)	1.5 L/ha (750 g terbuthylazine/ha)
Rate of Use (Min)	1.0 L/ha (500 g terbuthylazine/ha)
Physical state	Liquid
Formulation type	Suspension Concentrate (SC)
Batch number	1/23
Manifacturing date	January 2023
Expiry date	January 2025
Storage conditions	Ambient temperature

Equipment and apparatus

- Standard laboratory glassware/equipment
- Analytical Balance, OHAUS, PX224 Pioneer
- Technical balance accurate to 0.01 g, Sartorius, BP 3100 P
- HPLC equipped with DAD detector, Agilent, 1290 Infinity II
- LC with TRIPLE TOF 4600 AB SCIEX detector, Agilent ABSCIEX, HPLC Series 1290 TRIPLE TOF 4600
- Chromatographic column 250*4.6 mm *5µm particle size, Thermoscientific, Hypersil ODS
- Single use syringe with PTFE filter 0.45 µm pre size, Scharlab, PTFE
- Ultrasonic bath, AGE, Uniset AC
- Hydrometer (Series)

Instrument conditions see point 5.2.1.1.

Validation - Results and discussions

Table 5.2-2: Methods suitable for the determination of relevant impurities in plant protection product H-01-2022

	Atrazine (max. content 1 g/kg)	**Propazine (max. content 9 g/kg)**	**Simazine (max. content 9 g/kg)**
Author(s), year	Condorelli A.M.M., 2023		
Principle of method	HPLC-DAD		
Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)	The linearity of the analytical method was assessed using eight standard solutions in the concentration range: 0.0036-0.0505 mg/mL (0.2549 – 3.5692 g/kg tech. material) y=98662,3958x-6.5644 Correlation coefficient: R^2 = 0.9999	The linearity of the analytical method was assessed using eight standard solutions in the concentration range: 0.0095-0.1329 mg/mL (0.6718 – 9.4052 g/kg tech. material) y=90030,3288x-2.7703 Correlation coefficient: R^2 = 0.9999	The linearity of the analytical method was assessed using eleven standard solutions in the concentration range: 0.0013 - 0.1303 mg/mL (0.0922 – 9.2192 g/kg tech. material) y=100795.5405x-15.2939 Correlation coefficient: R^2 = 0.9996
Precision – Repeatability Mean n = 5 (%RSD)	0.024% w/w (LOQ) RSD % = 3.85 Horowitz %:4.70 Hr = 0.82 Acceptability: Hr ≤ 1	0.06% w/w (LOQ) RSD % = 3.80 Horowitz %:4.06 Hr = 0.94 Acceptability: Hr ≤ 1	0.011% w/w (LOQ) RSD % = 4.17 Horowitz %:5.29 Hr = 0.79 Acceptability: Hr ≤ 1
Accuracy n = 5 LOQ; n=2 (% Marginal Recovery)	LOQ level: 0.024% w/w 0.1205% w/w (n=2) 85.1% Acceptability: 80% - 120%	LOQ level: 0.06% w/w 0.32% w/w (n=2) 88.8% Acceptability: 80% - 120%	LOQ level: 0.011% w/w 0.310% w/w (n=2) 103.1% Acceptability: 80% - 120%
Interference/ Specificity	No interference. Acceptability: Interference <3% of analyte	No interference. Acceptability: Interference <3% of analyte	No interference. Acceptability: Interference <3% of analyte
LOQ	0.0072 mg/mL (0.024% w/w)	0.0190 mg/mL (0.06% w/w)	0.0033 mg/mL (0.011% w/w)
Comment	No comments.	No comments.	No comments.

Conclusion

A HPLC-DAD method was validated for determination of relevant impurities. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

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

Not relevant. The product H-01-2022 does not contain materials of toxicological, ecotoxicological or environmental concern.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

For the determination of terbuthylazine the CIPAC method 234/TC/M/3 is available.

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 terbuthylazine for the generation of pre-authorization data is given in the following table. The detailed evaluation of additional studies, it is referred to Appendix 2.

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

Component of residue definition: terbuthylazine				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Soil (Predatory mite *Hypoaspis (Geolaelaps) aculeifer* reproduction test) (Ecotoxicology)	Primary & confirmatory	0.05 mg/kg	HPLC-MS/MS	Mautino G., 2023 / Report No.: 1015.1H.SAG23 / 23133-01R
Soil (Nitrogen transformation test) (Ecotoxicology)	Primary	233.97 µg/mL	HPLC with DAD and PC detection	Kiran Yadav C., 2023 - Report No.: AG-G1155
Reconstituted water (*Daphnia magna*, Acute Immobilization Test) (Ecotoxicology)	Primary	46.7125 µg/L	LC-MS/MS	Likith N.G., 2023 / Report No.: AG-G1146
Algal water (*Raphidocelis subcapitata* Growth Inhibition Test) (Ecotoxicology)	Primary	47.1750 µg/L	LC-MS/MS	Likith N.G., 2023 / Report No.: AG-G1147
Lemna water (*Lemna* Growth	Primary	47.1750 µg/L	LC-MS/MS	Likith N.G., 2023 / Report No.: AG-G1148

Component of residue definition: terbuthylazine				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Inhibition Test) (Ecotoxicology)				
Artificial soil (*Eisenia fetida* Earthworm Reproduction Test) (Ecotoxicology)	Primary	0.00463 mg/kg	LC-MS/MS	Vishala N., 2023 / Report No.: AG-G1153
Smart and Barko Medium (water samples), sediment and sediment pore water (*Myriophyllum Spicatum* Water Sediment Toxicity Test) (Ecotoxicology)	Primary	0.0101 mg/L (Smart and Barko medium) 0.0098 mg/g (Sediment) 0.0104 mg/L (Sediment pore water)	LC-MS/MS	Likith N.G., 2023 / Report No.: AG-G1158
Milli-Q water (Honeybee (*Apis mellifera* L) larval toxicity test, repeated exposure) (Ecotoxicology)	Primary	0.051 µg/µL	LC-MS/MS	Gangadhar R. S., 2024 / Report No.: AG-G1149
50% w/v Sucrose solution in Milli-Q water (Honeybee (*Apis mellifera* L) chronic oral toxicity test) (Ecotoxicology)	Primary	0.051 mg/kg	LC-MS/MS	Gangadhar R. S., 2024 / Report No.: AG-G1152
Deionized water (Seedling emergence and seedling growth test with terrestrial plants) (Ecotoxicology)	Primary	2.5879 mg/L	HPLC with UV/PDA detector	Vishala N., 2023 / Report No.: AG-G1156
Deionized water (Vegetative vigour test) (Ecotoxicology)	Primary	2.5879 mg/L	HPLC with PDA detector	Vishala N., 2023 / Report No.: AG-G1157

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

5.3.1 Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substance and relevant impurities in the plant protection product are already submitted in accordance with the requirements set out in point 5.2.1 and can be applied.

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

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

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

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

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Plant, high water content	Terbuthylazine (MT0)	0.01 mg/kg	Regulation (EU) No. 2021/1795
Plant, high acid content		0.01 mg/kg	
Plant, high protein/high starch content (dry commodities)		0.01 mg/kg	
Plant, high oil content		0.01 mg/kg	
Muscle	Not necessary for the representative uses (EFSA Journal 2017;15(6):4868)*	-	-
Milk		-	
Eggs		-	
Fat		-	
Liver, kidney		-	
Soil (Ecotoxicology)	Terbuthylazine (MT0) plus desethyl-terbuthylazine (MT1) plus hydroxyl-terbuthylazine (MT13)	0.05 mg/kg	common limit
Drinking water (Human toxicology)	Terbuthylazine (MT0) plus desethyl-terbuthylazine (MT1) plus hydroxy-terbuthylazine (MT13) plus desethyl-hydroxy-terbuthylazine (MT14) plus LM1, LM2, LM3, LM4, LM5 and LM6	0.1 µg/L	general limit for drinking water
Surface water (Ecotoxicology)	Terbuthylazine (MT0) plus desethyl-terbuthylazine (MT1) plus hydroxyl-terbuthylazine (MT13)	E_bC_{50}: 0.012 mg a.s./L *Pseudokirchneriella subcapitata*	EFSA Journal 2017;15(6):4868

Matrix	Residue definition	MRL / limit	Reference for MRL/level Remarks
Air	Terbuthylazine	0.96 µg/m^3 (AOEL sys = 0.0032 mg/kg bw/day)	Calculated according to SANTE/2020/12830, Rev.2 14. February 2021
Tissue (meat or liver)	Not required	0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023
Body fluids		0.01 mg/kg	SANTE/2020/12830, Rev.2 14. February 2023

* According to EFSA Journal 2020;18(1):5980: *Based on the results of the metabolism study on ruminants, the following residue definition is proposed in milk for enforcement: sum of terbuthylazine and MT1, expressed as terbuthylazine. As no residues are expected in this commodity at the calculated dietary burden, the MRL can be derived at the LOQ.*
For all other tissues, in the absence of a full characterization of the TRR in the available metabolism study, it is not possible to derive a residue definition for enforcement and risk assessment nor MRL proposals.

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

zRMS comment
EFSA Journal 2020;18(1):5980:
Methods of analysis for monitoring of residues (analytical technique, matrix groups, LOQs):
Dry and high-water content matrices: GC-NPD, LOQ 0.02mg/kg (DFG S19) for terbuthylazine in cereal grain, ILV considered acceptable (EF-SA, 2011). This method was reported validated for maize whole plant during the peer however an ILV and confirmatory methods are missing (United Kingdom, 2007; Spain, 2018)
High oil content matrices: LC–MS/MS method for terbuthylazine, MT1 and MT14 with an individual LOQ of 0.02 mg/kg; ILV and confirmatory method are missing (United Kingdom, 2010a; Spain, 2018)

EURLs (EURLs, 2018) provided for routine analyses the following methods for terbuthylazine: High water, acid content commodities and high oil commodities: •LC–MS/MS method (QuEChERS-method EN 15662:2008) with a LOQ 0.01 mg/kg, validated in tomato, orange, almonds and avocado; Dry matrices: •LC-QqQ-MS/MS method (QuEChERS-method EN 15662:2008) with a LOQ 0.01 mg/kg validated in wheat, oat, rice and rye; Special matrices: •LC–MS/MS method (QuEChERS-method EN 15662:2008) with a LOQ 0.01 mg/kg, validated in green tea.

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

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

Component of residue definition: terbuthylazine				
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	0.02 mg/kg	GC-NPD DFG S7	Anon., 1987/Additional Report to DAR, 2010
	ILV	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
	Confirmatory (if required)	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
High acid content	Primary	0.02 mg/kg	GC-NPD DFG S7	Anon., 1987/Additional Report to DAR, 2010
	ILV	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
	Confirmatory (if required)	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
High oil content	Primary	0.02 mg/kg	GC-NPD DFG S7	Anon., 1987/Additional Report to DAR, 2010
	ILV	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
	Confirmatory (if required)	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
High protein/high starch content (dry)	Primary	0.02 mg/kg	GC-NPD DFG S7	Dierterle R., 1993/Additional Report to DAR, 2010
	ILV	0.02 mg/kg	GC-MS REM 201.01	Ferguson L., 2009/Additional Report to DAR, 2010
	Confirmatory (if required)	0.02 mg/kg	GC-MS REM 201.01	Anon., 1987/Additional Report to DAR, 2010

Table 5.3-3: Statement on extraction efficiency

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

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

According to EFSA Journal 2020;18(1):5980:

Methods of analysis for monitoring of residues (analytical technique, matrix groups, LOQs): LC–MS/MS multi-residue QuEChERS method (EN 15662:2009-02) for terbuthylazine in animal matrices; LOQ 0.01mg/kg; confirmatory method and ILV available for milk, meat, egg, liver, fat and kidney; LOD <0.00125 mg/kg (Spain, 2018) Analytical method for enforcement of MT1 in milk, not available and required. GC-NPD, LOQ 0.02mg/kg (DFG S19) for terbuthylazine in animal matrices; confirmatory method missing and ILV available (United Kingdom, 2007) EURLs (EURLs, 2018) provided for routine analyses of food of animal origin for terbuthylazine an:

- *LC–MS-Q-TOF QuEChERS with a screening detection limit (SDL) of 0.0025 mg/kg for terbuthylazine, validated in milk and milk products; meat (red and white), fish (high and low fat), various honeys,*
- *LC–MS/MS QuEChERS EN-15662 method for monitoring terbuthylazine and MT1 with an individual LOQ of 0.01 mg/kg in milk (EURLs, 2018).*

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

Table 5.3-4: Validated methods for soil

Component of residue definition: terbuthylazine			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.02 mg/kg	GC-NPD REM 148.05	Lutolf W., 1995a/Additional Report to the DAR, 2010
Confirmatory	0.02 mg/kg	GC-MS REM 148.05	Lutolf W., 1995a/Additional Report to the DAR, 2010
Primary	0.02 mg/kg	HPLC-MS/MS REM 148.11	Fugueiredo J., 2003 /Additional Report to the DAR, 2010 Tribolet R., 2003 /Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Primary	0.01 mg/kg	HPLC-MS/MS	Todd. M, 2002a/Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Component of residue definition: MT1			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.02 mg/kg	HPLC-MS/MS REM 148.11	Fugueiredo J., 2003 /Additional Report to the DAR, 2010 Tribolet R., 2003

Component of residue definition: terbuthylazine			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
			/Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Primary	0.01 mg/kg	HPLC-MS/MS	Todd. M, 2002a/Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Component of residue definition: MT13			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.02 mg/kg	HPLC-MS/MS REM 148.11	Fugueiredo J., 2003 /Additional Report to the DAR, 2010 Tribolet R., 2003 /Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Primary	0.01 mg/kg	HPLC-MS/MS	Todd. M, 2002a/Additional Report to the DAR, 2010
Confirmatory	Not required	-	-
Component of residue definition: MT14			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	0.02 mg/kg	HPLC-MS/MS REM 148.11	Fugueiredo J., 2003 /Additional Report to the DAR, 2010 Tribolet R., 2003 /Additional Report to the DAR, 2010
Confirmatory	Not required	-	-

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

Table 5.3-5: Validated methods for water

Component of residue definition: terbuthylazine, MT1, MT13

Matrix type	Method type	Method LOQ	Principle of method (i.e., GC-MS or HPLC-UV)	Author(s), year / missing
Drinking water	Primary	0.1 µg/L	HPLC-MS/MS RAM 426/01	Robinson N J., 2004/Additional Report to the DAR, 2010
	ILV	Not available. This data gap is anticipated to be addressed at active substance level in context with the renewal of terbuthylazine.		
	Confirmatory	Not required.		
Surface water	Primary	0.1 µg/L	HPLC-MS/MS RAM 426/01	Robinson N J., 2004/Additional Report to the DAR, 2010
	Confirmatory	Not required.		
Drinking water	Primary	0.05 µg/L	HPLC-MS/MS	Todd M., 2002b/Additional Report to the DAR, 2010
	ILV	Not available. This data gap is anticipated to be addressed at active substance level in context with the renewal of terbuthylazine.		
	Confirmatory	Not required.		
Surface water	Primary	0.05 µg/L	HPLC-MS/MS	Todd M., 2002b/Additional Report to the DAR, 2010
	Confirmatory	Not required.		
Component of residue definition: MT14				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.1 µg/L	HPLC-MS/MS RAM 426/01	Robinson N J., 2004/Additional Report to the DAR, 2010
	ILV	Not available. This data gap is anticipated to be addressed at active substance level in context with the renewal of terbuthylazine.		
	Confirmatory	Not required.		
Surface water	Primary	0.1 µg/L	HPLC-MS/MS RAM 426/01	Robinson N J., 2004/Additional Report to the DAR, 2010
	Confirmatory	Not required.		
Component of residue definition: LM3, LM5, LM6				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Drinking water	Primary	0.05 µg/L	HPLC-MS/MS GRM015.02	Zietz, E., 2009 and 2009b/Additional Report to the DAR, 2010
	ILV	Not available. This data gap is anticipated to be addressed at active substance level in context with the renewal of terbuthylazine.		

Component of residue definition: terbuthylazine, MT1, MT13				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
	Confirmatory	Not required.		
Surface water	Primary	0.05 µg/L	HPLC-MS/MS GRM015.02	Zietz, E., 2009 and 2009b/Additional Report to the DAR, 2010
	Confirmatory	Not required.		

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

Table 5.3-6: Validated methods for air

Component of residue definition: terbuthylazine			
Method type	**Method LOQ**	**Principle of method (i.e., GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	1 µg/m^3	GC-NPD	Tribolet R., 1992/Additional Report to the DAR, 2010
Confirmatory	1 µg/m^3	GC-MSD	Tribolet R., 1992/Additional Report to the DAR, 2010
Primary	0.5 µg/m^3	GC-NPD	Schultz M., and Ullrich-Mitzel A., 1995/Additional Report to the DAR, 2010

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

In DAR for terbuthylazine, 2007 (incl. Additional Report, 2010) and EFSA Journal 2017;15(6):4868 methods for body fluids and tissues are not required and are not available. However, according to the Regulation No. 283/2013 and to the SANTE/2020/12830, Rev.2, 14. February 2023 an analytical method for the determination of residues in body fluids and tissues for enforcement/monitoring purposes is required.
In Applicant opinion no additional studies are necessary and this data gap should be addressed at active substance level in context with the renewal of terbuthylazine.

5.3.2.8 Other studies/ information

Not necessary.

Appendix 1 Lists of data considered in support of the evaluation

Tables considered not relevant can be deleted as appropriate.

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

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	Owner
KCP 5.1.1/01 KCP 5.1.1/02	Condorelli A.M.M.	2023	Analytical Method Validation for Active Ingredient and impurities Content Determination of the H-01-2022 product in Order to Provide an Analytical Certificate Report No. 22363-01C Renolab S.r.l. GLP Published	N	ProAgri Sp. z o. o.
KCP 5.1.2/01	Mautino G.	2023	Predatory mite *Hypoaspis (Geolaelaps) aculeifer* reproduction test in soil with H-01-2022 (terbuthylazine 500 g/L) - Analytical Phase Report No.: 1015.1H.SAG23 / 23133-01R Renolab S.r.l. GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/02 filled as KCP 10.5/01	Kiran Yadav C.	2023	SOIL MICROORGANISMS: NITROGEN TRANSFORMATION TEST OF H-01-2022 Report No.: AG-G1155 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/03 (filed as KCP 10.2.1.2/01)	Likith N.G.	2023	H-01-2022: *Daphnia magna*, ACUTE IMMOBILIZATION TEST Report No.: AG-G1146 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP	N	ProAgri International Sp. z o.o.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			Unpublished		
KCP 5.1.2/04 (filed as KCP 10.2.1.3/01)	Likith N.G.	2023	H-01-2022: ALGA GROWTH INHIBITION TEST WITH *Raphidocelis subcapitata* Report No.: AG-G1147 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/05 (filed as KCP 10.2.1.4/01)	Likith N.G.	2023	H-01-2022: *LEMNA* GROWTH INHIBITION TEST Report No.: AG-G1148 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/06 (filed as KCP 10.4.1.1/01)	Vishala N.	2023	H-01-2022: EARTHWORM REPRODUCTION TEST *(Eisenia fetida)* Report No.: AG-G1153 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/07 (filed as KCP 10.2.1.4/02)	Likith N.G.	2023	H-01-2022: WATER SEDIMENT *MYRIOPHYLLUM SPICATUM* TOXICITY TEST Report No.: AG-G1158 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/08 (filed as KCP 10.3.1.4/01)	Gangadhar R. S.	2024	H-01-2022: HONEYBEE (*Apis mellifera* L) LARVAL TOXICITY TEST, REPEATED EXPOSURE Report No.: AG-G1149 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/09 (filed as KCP 10.3.1.1.2/02)	Gangadhar R. S.	2024	H-01-2022: CHRONIC ORAL TOXICITY TEST IN HONEYBEE (*Apis mellifera* L.) Report No.: AG-G1152 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED	N	ProAgri International Sp. z o.o.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
			GLP Unpublished		
KCP 5.1.2/10 (filed as KCP 10.6.2/01)	Vishala N.	2023	H-01-2022: SEEDLING EMERGENCE AND SEEDLING GROWTH TEST WITH TERRESTRIAL PLANTS Report No.: AG-G1156 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.
KCP 5.1.2/11 (filed as KCP 10.6.2/02)	Vishala N.	2023	H-01-2022: VEGETATIVE VIGOUR TEST Report No.: AG-G1157 EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED GLP Unpublished	N	ProAgri International Sp. z o.o.

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	Anon.	1987	S7 multi-method for Triazine Herbicides, DFG Deutsche Forschungsgemeinschaft, Manual of Pesticide Residue Analysis Volume 1. Pesticides Commission. DFG Deutsche Forschungsgemeinschaft, Manual of Pesticide Residue Analysis Volume 1. Pesticides Commission. DFG Deutsche Forschungsgemeinschaft, Manual of Pesticide Residue Analysis Volume 1. Pesticides Commission. Published Syngenta File N° N/0862	N	-
KCP 5.2	Ferguson L.	2009	Terbuthylazine – Independent Laboratory Validation of Analytical Method No. REM 201.01 for the	N	Oxon /

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
			Determination of Terbuthylazine (GS13529) and its Metabolites GS26379 and GS28620 in Whole Maize Plants and Rape Seed Syngenta - Jealott's Hill, Bracknell, United Kingdom; Oxon Italia, S.p.A, Pero, Italy Charles River Laboratories, Edinburgh, United Kingdom, Report No 30377 GLP Not published Syngenta File No GS13529_10121		Syngenta
KCP 5.2	Dieterle, R.	1993	GS 13529, Applicability of Multiresidue Method DFG S 19 for Determination of GS 13529 in Maize (Grain and Whole Plant) Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 121-92 GLP Not Published Syngenta File N° GS13529/1080	N	Syngenta
KCP 5.2	Luetolf, W.	1995a	Determination of residues of parent compound by gas chromatography (GC), Soil Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No REM 148.05 GLP Not Published Syngenta File N° GS13529/1276	N	Syngenta
KCP 5.2	Figueiredo, J.	2003	Determination of GS13529 (Terbuthylazine) and its metabolites GS26379, GS28620, and GS23158 in Soil by LC-MS/MS. REM 148.11. Syngenta Crop Protection AG, Basel, Switzerland, Report No REM 148.11 Not GLP Not Published Syngenta File N° GS13529/1835	N	Syngenta

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	Todd M.	2002a	Validation Of Methodology For The Post-Registration Monitoring Of Residues Of Terbuthylazine And Its Two Major Metabolites Desethyl Terbuthylazine And 2-Hydroxy Terbuthylazine In Soil Huntingdon Life Sciences Limited, Cambridgeshire, UK Oxon Italia S.P.A, Pero, Italy Report-no. OXN 228/024125 GLP Not Published	N	Oxon
KCP 5.2	Tribolet R.	2003	Validation of Method REM 148.11 by Analisys of Fortified Soil Specimens for Terbuthylazine (GS13529) and its Metabolites GS26379, GS28620 and GS23158 and Evaluation of Recoveries. Syngenta Crop Protection AG, Basel, Switzerland Report No 02-S310 GLP Not Published Syngenta File N° GS13529/1836	N	Syngenta
KCP 5.2	Robinson, N. J.	2004	Residue Analytical Method for the Determination of Residues of Terbuthylazine (GS13529), GS23158, GS26379 and GS28620 in Water Syngenta Crop Protection AG, Basel, Switzerland Syngenta, Jealott's Hill, United Kingdom, Report No RAM 426/01 GLP Not Published Syngenta File N° GS13529/1916	N	Syngenta
KCP 5.2	Todd M.	2002b	Terbuthylazine: Validation Of Methodology For The Determination Of Residues Of Terbuthylazine And Its Two Major Metabolites Desethyl Terbuthylazine And 2-Hydroxy Terbuthylazine In Drinking And Surface Water Huntingdon Life Sciences Limited, Cambridgeshire, UK Oxon Italia S.P.A, Pero, Italy Report-no. OXN 229/024126 GLP Not Published	N	Oxon

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.2	Zietz E.	2009	Terbuthylazine - Validation of an Analytical Method (Draft GRM015.02A) for the Determination of Residues of the Terbuthylazine Metabolites CSCD648241 and GS16984 in Groundwater, Surface Water, and Drinking Water Syngenta - Jealott's Hill, Bracknell, United Kingdom; Oxon Italia, S.p.A, Pero, Italy SGS Institut Fresenius GmbH, D-65232 Taunusstein, Germany IF 08/01259634, T000964-09 GLP Not Published Syngenta File No GS13529_10092	N	Oxon / Syngenta
KCP 5.2	Tribolet, R.	1992	Sampling of air and determination of residues of parent compound by gas chromatography Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No REM-148-03 GLP Not Published Syngenta File N° GS13529/1057	N	Syngenta
KCP 5.2	Schulz M., Ullrich-Mitzel A.	1995	Analytical Method For The Determination Of Terbuthylazine In Air RCC AG., Itingen, Switzerland Oxon Italia S.P.A, Pero, Italy Report-no. 385615 GLP Not Published	N	Oxon

The following tables are to be completed by MS

List of data submitted by the applicant and not relied on

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

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

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

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for terbuthylazine

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

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

A 2.1.1.1.1.1 Method validation

Comments of Evaluator:	The method was evaluated in section 5.2.

Reference:	5.1.1/01
Report	Analytical Method Validation for Active Ingredient and impurities Content Determination of the H-01-2022 product in Order to Provide an Analytical Certificate, Condorelli A.M.M., 2023, report no. 22363-01C
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study objective is the Analytical Method Validation for Active Ingredient and impurities content Determination of the H-01-2022 product in Order to fulfil to the requirements of Regulation (EC) No. 1107/2009 and commission implementing Regulation (EU) No 2021/824. A HPLC-DAD method was validated for determination of terbuthylazine and its relevant impurities in H-01-2022 product.

Test item

Name	H-01-2022
Active substance	Terbuthylazine
IUPAC name of a.s.	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine
CAS Number of a.s.	5915-41-3
Nominal content:	500 g terbuthylazine/L
Rate of Use (Max)	1.5 L/ha (750 g terbuthylazine/ha)
Rate of Use (Min)	1.0 L/ha (500 g terbuthylazine/ha)
Physical state	Liquid
Formulation type	Suspension Concentrate (SC)
Batch number	1/23
Manufacturing date	January 2023
Expiry date	January 2025
Storage conditions	Ambient temperature

Equipment and apparatus

- Standard laboratory glassware/equipment
- Analytical Balance, OHAUS, PX224 Pioneer
- Technical balance accurate to 0.01 g, Sartorius, BP 3100 P
- HPLC equipped with DAD detector, Agilent, 1290 Infinity II
- LC with TRIPLE TOF 4600 AB SCIEX detector, Agilent ABSCIEX, HPLC Series 1290 TRIPLE TOF 4600
- Chromatographic column 250*4.6 mm *5µm particle size, Thermoscientific, Hypersil ODS
- Single use syringe with PTFE filter 0.45 µm pre size, Scharlab, PTFE
- Ultrasonic bath, AGE, Uniset AC
- Hydrometer (Series)

Reagents and Materials

- Acetonitrile for HPLC, Merck
- Ultrapure water for HPLC, Merck
- Methanol for GC, Merck
- Formic Acid, Merck

Test Item Solutions Preparation

Test item was accurately weighed into a class A volumetric flask, then made up to volume with acetonitrile and opportunely diluted to reach concentration of 0.2 mg/mL and analysed by HPLC-DAD.
Five replicates were prepared for method validation and determination.
Blank formulation was accurately weighed into a class A volumetric flask then made up to volume with acetonitrile to reach the same concentration as test item.

Reference Item Solutions Preparation

Taking into account the analytical standard purity, two sets of reference item stock solutions in acetonitrile were prepared as follows:

Reference item		Purity [%]	Weight [mg]	Final Volume [mL]	Concentration [mg/mL]	Code
Id. Code and batch	Operation n°.					
SR 263 Batch BCCF6584	12	97.8	21.9	20	1.0709	SM 263-12
	13	97.8	27.2	20	1.3301	SM 263-13

Reference item working solutions used in the calibration and determination of Terbuthylazine

Id Code	Initial Conc.	Volume taken	Final volume	Final Conc.[1]	% w/w [2]	Id. Code
	[mg/mL]	[mL]	[mL]	[mg/mL]	[%]	
SM 263-12	1.0709	0.4	10	0.0428	21.4	SL 263-12 A
SM 263-12	1.0709	0.7	10	0.0750	37.5	SL 263-12 B
SM 263-12	1.0709	0.9	10	0.0964	48.2	SL 263-12 C
SM 263-12	1.0709	0.5	5	0.1071	53.5	SL 263-12 D
SM 263-12	1.0709	0.7	5	0.1499	75.0	SL 263-12 E
SM 263-13	1.3301	0.9	10	0.1197	59.9	SL 263-13 A (QC)

[1]: Reference item solution concentration is calculated as $\frac{Stock\ solution\ concentration \cdot Volume\ taken}{Final\ volume}$

[2]: % w/w (analyte weight/sample weight) based on nominal test item solution concentration of 0.2 mg/mL, calculated as $\frac{Working\ solution\ concentrati \cdot 100}{Nominal\ test\ item\ solution\ concentration}$

Validation - Results and discussions

Specificity

The chromatogram and the retention time of active ingredients in the sample solution matched the chromatogram, the retention time and spectrum in the reference standard solution.

Linearity

The linear range of the detector response was determined by injecting the standard solutions shown on tables above. The detector response was linear for the active ingredient in the range tabled below, with the following calibration line (y = 83,817.1929x - 62.7218):

Active ingredient	Conc. Range	Calibration function		Square Correlation Coefficient [r^2]
		Slope	Intercept	
Terbuthylazine	0.0428-0.1499 mg/mL 21.4-75.0 %w/w	83817.19	-62.72	0.9993

Precision (Repeatability)

Independent test item solutions were prepared as described in above and analysed with the HPLC-DAD method above. The results were summarized in below.

Sample ID	Area	Conc.	Weight	Sample volume* Dilution	AI content	AI content
[-]	[mAU*s]	[mg/mL]	[mg]	[mL]	[%]	[g/L]
22363-1 Dil nb	8863.892	0.1065	118.6	500	44.9	502
22363-2 Dil nb	7324.021	0.0881	98.8	500	44.6	499
22363-3 Dil nb	8615.484	0.1035	115.7	500	44.7	501
22363-4 Dil nb	9014.38	0.1083	120.2	500	45.0	504
22363-5 Dil nb	7860.234	0.0945	106.2	500	44.5	498
MEAN					**44.8**	**501**
DEV.ST					**0.2**	**2**
RSD %					**0.49**	**0.49**
RSD r %					**1.51**	**1.05**
Horrat					**0.33**	**0.47**

Where:
% RSDr = 0,67*2(1-0,5*log(c)) called Horwitz equation
Hr = %RSD/%RSDr called Horwitz ratio, acceptability according to SANCO 3030/99 rev. 5, HR <1

Accuracy

To determine the accuracy of the analytical method, recovery experiments for the active substance were performed. Volumes of active ingredient reference item stock solution were added to the placebo, at level

of the nominal concentration of active ingredient in the prepared solution and determined in accordance with the analytical method.

The accuracy of the analytical method was reported as Mean Recovery (%) ± Relative Standard Deviation

As acceptability criteria, a Mean Recovery of 97-103 % was adopted.

Accuracy results for Terbuthylazine

Recovery solution Code	Area [mAU*s]	C_F [mg/mL]	C_U [mg/mL]	C_A [mg/mL]	Recovery [%]	Mean recovery [%]
REC1 nb	7735.713	0.0930	0.0883	0.0964	101.5	101.5
REC2 nb	8370.33	0.1006	0.1035	0.0964	101.4	

Conclusion

A HPLC-DAD method was validated for determination of terbuthylazine. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

A 2.1.1.1.1.2 Method validation

Comments of Evaluator:	The method was evaluated in section 5.2.

Reference:	5.1.1/02
Report	Analytical Method Validation for Active Ingredient and impurities Content Determination of the H-01-2022 product in Order to Provide an Analytical Certificate, Condorelli A.M.M., 2023, report no. 22363-01C
Guideline(s):	Yes, SANCO/3030/99 rev.5
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The study objective is the Analytical Method Validation for Active Ingredient and impurities content Determination of the H-01-2022 product in Order to fulfil to the requirements of Regulation (EC) No 1107/2009 and commission implementing Regulation (EU) No 2021/824. A HPLC-DAD method was validated for determination of terbuthylazine and its relevant impurities in H-01-2022 product.

Test item

Name	H-01-2022
Active substance	Terbuthylazine
IUPAC name of a.s.	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine
CAS Number of a.s.	5915-41-3
Nominal content:	500 g terbuthylazine/L
Rate of Use (Max)	1.5 L/ha (750 g terbuthylazine/ha)
Rate of Use (Min)	1.0 L/ha (500 g terbuthylazine/ha)

Physical state	Liquid
Formulation type	Suspension Concentrate (SC)
Batch number	1/23
Manifacturing date	January 2023
Expiry date	January 2025
Storage conditions	Ambient temperature

Equipment and apparatus

- Standard laboratory glassware/equipment
- Analytical Balance, OHAUS, PX224 Pioneer
- Technical balance accurate to 0.01 g, Sartorius, BP 3100 P
- HPLC equipped with DAD detector, Agilent, 1290 Infinity II
- LC with TRIPLE TOF 4600 AB SCIEX detector, Agilent ABSCIEX, HPLC Series 1290 TRIPLE TOF 4600
- Chromatographic column 250*4.6 mm *5µm particle size, Thermoscientific, Hypersil ODS
- Single use syringe with PTFE filter 0.45 µm pre size, Scharlab, PTFE
- Ultrasonic bath, AGE, Uniset AC
- Hydrometer (Series)

Reagents and Materials

- Acetonitrile for HPLC, Merck
- Ultrapure water for HPLC, Merck
- Methanol for GC, Merck
- Formic Acid, Merck

Test Item Solutions Preparation

Test item was weighted in a volumetric class A flask and made up to volume with acetonitrile to reach a concentration of 30 mg/mL. Samples were accurately homogenised by vigorous hand shaking, sonicated for 10 minutes and filtered with PTFE filters.

Atrazine

Reference Item Solutions Preparation

Taking into account the analytical standard purity, two set of reference item stock solution in solvent mixture water-acetonitrile was prepared as follows. Standard solutions used for linearity have been prepared as mix solution of Atrazine, Simazine and Propazine.

Stock solutions for Atrazine determination

Stock solution Id. code and batch	Purity [%]	Weight [mg]	Volume [mL]	Conc. [mg/mL]	Id. Code
SR 521 Batch BCCH0564	99.00	18.2	50	0.3604	SM 521-6
	99.00	18.7	100	0.1851	SM 521-7

From the above stock solution were prepared the following reference working solutions for linearity.

Reference item working solutions for Atrazine determination

Id Code	Initial Conc.	Volume	Final volume	Final Conc.[1]	g/Kg referred	g/L referred	Id. Code

		taken			to technical material[2]	to test Item[3]	
	[mg/mL]	[mL]	[mL]	[mg/mL]	[g/kg]	[g/L]	
SM 521-6	0.3604	0.20	20	0.0036	0.2549	0.1344	SL mix A
SM 521-6	0.3604	0.20	10	0.0072	0.5099	0.2688	SL mix B
SM 521-6	0.3604	0.30	10	0.0108	0.7648	0.4032	SL mix C
SM 521-6	0.3604	0.40	10	0.0144	1.0198	0.5377	SL mix D
SM 521-6	0.3604	0.50	10	0.0180	1.2747	0.6721	SL mix E
SM 521-6	0.3604	0.60	10	0.0216	1.5297	0.8065	SL mix F
SM 521-6	0.3604	1.00	10	0.0360	2.5495	1.3441	SL mix G
SM 521-6	0.3604	1.40	10	0.0505	3.5692	1.8818	SL mix H
SM 521-7	0.1851	0.50	10	0.0093	0.6549	0.3453	SL mix QC

[1]: Reference item solution concentration is calculated as $\frac{Stock\ solution\ concentration \cdot Volume\ taken}{Final\ volume}$

[2]: **g/Kg referred to technical material** based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%; calculated as

$$\frac{\text{Final Conc} * \%\text{purity of technical material} * 1000}{\text{Nominal test item solution concentration} * \text{Content of AI \%}}$$

[3]: **g/L referred to the test item** based on nominal test item solution concentration of 30 mg/mL, Density of test item of 1.119 g/mL; calculated as

$$\frac{\text{Final Conc} * \text{density} * 1000}{\text{Nominal test item solution concentration}}$$

Validation - Results and discussions

Specificity

The specificity for all impurities was assessed with HPLC-MS/MS technique. Solutions for every standard and test item were analysed with HPLC-MS/MS instrument for specificity analysis. Solutions were prepared by diluting the sample in Acetonitrile.

Linearity

The linear range of the detector response was determined for each impurity by injecting the standard solutions described above. Range of calibration parameters and correlation coefficient of the curves are reported below (y = 98,662.3958x - 6.5644):

Parameters of the linearity curve for Atrazine

Lower point	Higher point	a parameter (y = ax +b)	b parameter (y = ax + b)	R^2
0.0036 mg/mL (0.2549 g/Kg tech material) [1]	0.0505 mg/mL (3.5692 g/Kg tech material) [1]	98662.40	-6.56	0.9999

[1] based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%.

Precision (Repeatability)

Independent test item solutions were prepared as described in above and analysed with the HPLC-UV method above. The results were summarized in below.

Summary of Content for Atrazine

Sample No.	Test item weight	Volume	Dilution factor	Content
-	[g]	[mL]	-	[mg/Kg][1]
22363-imp 1	0.3026	10	1	below LOQ (0.5099 mg/kg of technical material)
22363-imp 2	0.2949	10	1	below LOQ (0.5099 mg/kg of technical material)
22363-imp 3	0.3093	10	1	below LOQ (0.5099 mg/kg of technical material)
22363- imp 4	0.3117	10	1	below LOQ (0.5099 mg/kg of technical material)
22363-imp 5	0.3108	10	1	below LOQ (0.5099 mg/kg of technical material)

[1] Referred to the Terbuthylazine technical material

Since the content found is <LOQ, precision is calculated on recovery analysis.

Accuracy

To determine the accuracy of the analytical methods, recovery experiments, were performed by fortification of test item. Recovery was prepared at two levels, LOQ and an appropriate second level set between the higher concentration found in test item and 80 per cent of the concentration of the last point in the calibration curve.

As acceptability criteria, a Mean Recovery of 80-120 % was adopted.

Accuracy results for Atrazine

Recovery solution Code	Area	C_F	C_U	C_A	Recovery	Mean recovery
[-]	**[mAU*s]**	**[mg/mL]**	**[mg/mL]**	**[mg/mL]**	**[%]**	**[%]**
REC LOQ 1	598.0	0.0061	0.0000	0.0072	85.0	**85.1**
REC LOQ 2	551.5	0.0057	0.0000	0.0072	78.5	
REC LOQ 3	614.3	0.0063	0.0000	0.0072	87.3	
REC LOQ 4	607.3	0.0062	0.0000	0.0072	86.3	
REC LOQ 5	623.2	0.0064	0.0000	0.0072	88.6	
REC MAX 1	2982.0	0.0303	0.0000	0.0360	84.1	**85.1**
REC MAX 2	3057.1	0.0311	0.0000	0.0360	86.2	

Limit of quantification (LOQ)

The limit of quantification (LOQ) according to SANCO/3030/99 rev. 5 is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable RSD is obtained.

LOQ was tested by injecting five distinct recovery tests.

Summary of precision of Atrazine at LOQ

Test item concentration	Recovery level at LOQ		Mean recovery	Standard dev.	RSD	RSDr	Horrat
[mg/mL]	**[mg/mL]**	**[% w/w]**	**[%]**	**[-]**	**[%]**	**[%]**	**[-]**
30	0.0072	0.024	85.1	3.28	3.85	4.70	0.82

Propazine

Reference Item Solutions Preparation

Taking into account the analytical standard purity, two set of reference item stock solution in solvent mixture water-acetonitrile was prepared as follows. Standard solutions used for linearity have been prepared as mix solution of Atrazine, Simazine and Propazine.

Stock solutions for Propazine determination

Stock solution Id. code and batch	Purity [%]	Weight [mg]	Volume [mL]	Conc. [mg/mL]	Id. Code
SR 522 Batch BCBX0853	99.12	47.9	50	0.9496	SM 522-5
	99.12	47.9	100	0.4748	SM 522-6

From the above stock solution were prepared the following reference working solutions for linearity.

Reference item working solutions for Propazine determination

Id Code	Initial Conc.	Volume taken	Final volume	Final Conc.[1]	g/Kg referred to technical material[2]	g/L referred to test Item[3]	Id. Code
	[mg/mL]	[mL]	[mL]	[mg/mL]	[g/kg]	[g/L]	
SM 522-5	0.950	0.20	20	0.0095	0.6718	0.3542	SL mix A
SM 522-5	0.950	0.20	10	0.0190	1.3436	0.7084	SL mix B
SM 522-5	0.950	0.30	10	0.0285	2.0154	1.0626	SL mix C
SM 522-5	0.950	0.40	10	0.0380	2.6872	1.4168	SL mix D
SM 522-5	0.950	0.50	10	0.0475	3.3590	1.7709	SL mix E
SM 522-5	0.9496	0.60	10	0.0570	4.0308	2.1251	SL mix F
SM 522-5	0.9496	1.00	10	0.0950	6.7180	3.5419	SL mix G
SM 522-5	0.9496	1.40	10	0.1329	9.4052	4.9587	SL mix H
SM 522-6	0.475	1.40	10	0.0665	4.7026	2.4793	SL mix QC

[1]: Reference item solution concentration is calculated as $\frac{Stock\ solution\ concentration \cdot Volume\ taken}{Final\ volume}$

[2]: **g/Kg referred to technical material** based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%; calculated as

$$\frac{\text{Final Conc} * \%\text{purity of technical material} * 1000}{\text{Nominal test item solution concentration} * \text{Content of AI \%}}$$

[3]: **g/L referred to the test item** based on nominal test item solution concentration of 30 mg/mL, Density of test item of 1.119 g/mL; calculated as

$$\frac{\text{Final Conc} * \text{density} * 1000}{\text{Nominal test item solution concentration}}$$

Validation - Results and discussions

Specificity

The specificity for all impurities was assessed with HPLC-MS/MS technique. Solutions for every standard and test item were analysed with HPLC-MS/MS instrument for specificity analysis. Solutions were prepared by diluting the sample in Acetonitrile.

Linearity

The linear range of the detector response was determined for each impurity by injecting the standard solutions described above. Range of calibration parameters and correlation coefficient of the curves are reported below (y = 90,030.3288x - 2.7703):

Parameters of the linearity curve for Propazine

Lower point	Higher point	a parameter (y = ax +b)	b parameter (y = ax + b)	R^2
0.0095 mg/mL (0.6718 g/Kg tech material) [1]	0.1329 mg/mL (9.4052 g/Kg tech material) [1]	90030.33	-2.77	0.9999

[1] based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%.

Precision (Repeatability)

Independent test item solutions were prepared as described in above and analysed with the HPLC-UV method above. The results were summarized in below.

Summary of Content for Propazine

Sample No.	Test item weight	Volume	Dilution factor	Content
-	[g]	[mL]	-	[mg/Kg][1]
22363-imp 1	0.3026	10	1	below LOQ (0.6718 mg/kg of technical material)
22363-imp 2	0.2949	10	1	below LOQ (0.6718 mg/kg of technical material)
22363-imp 3	0.3093	10	1	below LOQ (0.6718 mg/kg of technical material)
22363- imp 4	0.3117	10	1	below LOQ (0.6718 mg/kg of technical material)
22363-imp 5	0.3108	10	1	below LOQ (0.6718 mg/kg of technical material)

[1] Referred to the Terbuthylazine technical material

Since the content found is <LOQ, precision is calculated on recovery analysis.

Accuracy

To determine the accuracy of the analytical methods, recovery experiments, were performed by fortification of test item. Recovery was prepared at two levels, LOQ and an appropriate second level set between the higher concentration found in test item and 80 per cent of the concentration of the last point in the calibration curve.

As acceptability criteria, a Mean Recovery of 80-120 % was adopted.

Accuracy results for Propazine

Recovery solution Code	Area	C_F	C_U	C_A	Recovery	Mean recovery
[-]	[mAU*s]	[mg/mL]	[mg/mL]	[mg/mL]	[%]	[%]
REC LOQ 1	1450.1	0.0161	0.0000	0.0190	85.0	**88.8**
REC LOQ 2	1458.4	0.0162	0.0000	0.0190	85.5	
REC LOQ 3	1545.9	0.0172	0.0000	0.0190	90.6	

REC LOQ 4	1544.7	0.0172	0.0000	0.0190	90.5	
REC LOQ 5	1578.8	0.0176	0.0000	0.0190	92.5	
REC MAX 1	7492.9	0.0833	0.0000	0.0950	87.7	**88.6**
REC MAX 2	7658.6	0.0851	0.0000	0.0950	89.6	

Limit of quantification (LOQ)

The limit of quantification (LOQ) according to SANCO/3030/99 rev. 5 is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable RSD is obtained.

LOQ was tested by injecting five distinct recovery tests.

Summary of precision of Propazine at LOQ

Test item concentration	**Recovery level at LOQ**		**Mean recovery**	**Standard dev.**	**RSD**	**RSDr**	**Horrat**
[mg/mL]	**[mg/mL]**	**[% w/w]**	**[%]**	**[-]**	**[%]**	**[%]**	**[-]**
30	0.0190	0.06	88.8	3.38	3.80	4.06	0.94

Simazine

Reference Item Solutions Preparation

Taking into account the analytical standard purity, two set of reference item stock solution in solvent mixture acetone-acetonitrile was prepared as follows.

Stock solutions for Simazine determination

Stock solution	**Purity**	**Weight**	**Volume**	**Conc.**	**Id. Code**
Id. code and batch	**[%]**	**[mg]**	**[mL]**	**[mg/mL]**	
SR 523 Batch BCBX5661	98.81	47.1	50	0.9308	SM 523-9
	98.81	52.4	50	1.0355	SM 523-10

From the above stock solution were prepared the following reference working solutions for linearity.

Reference item working solutions for Simazine determination

Id Code	**Initial Conc.**	**Volume taken**	**Final volume**	**Final Conc.[1]**	**g/Kg referred to technical material[2]**	**g/L referred to test Item[3]**	**Id. Code**
	[mg/mL]	[mL]	[mL]	[mg/mL]	[g/kg]	[g/L]	
SL 523-9 H	0.1303	0.20	20	0.0013	0.0922	0.049	SL 523-9 AA
SL 523-9 H	0.1303	0.40	20	0.0026	0.1844	0.097	SL 523-9 AB
SL 523-9 H	0.1303	0.60	20	0.0039	0.2766	0.146	SL 523-9 AC
SM 523-9	0.9308	0.20	20	0.0093	0.6585	0.347	SL 523-9 A
SM 523-9	0.9308	0.20	10	0.0186	1.3170	0.694	SL 523-9 B
SM 523-9	0.9308	0.30	10	0.0279	1.9755	1.042	SL 523-9 C
SM 523-9	0.9308	0.40	10	0.0372	2.6341	1.389	SL 523-9 D

SM 523-9	0.9308	0.50	10	0.0465	3.2926	1.736	SL 523-9 E
SM 523-9	0.9308	0.60	10	0.0558	3.9511	2.083	SL 523-9 F
SM 523-9	0.9308	1.00	10	0.0931	6.5851	3.472	SL 523-9 G
SM 523-9	0.9308	1.40	10	0.1303	9.2192	4.861	SL 523-9 H
SM 523-10	1.0355	1.00	10	0.1036	7.3261	3.863	SL 523-10 QC

[1]: Reference item solution concentration is calculated as $\frac{Stock\ solution\ concentration \cdot Volume\ taken}{Final\ volume}$

[2]: **g/Kg referred to technical material** based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%; calculated as

$$\frac{\text{Final Conc} * \%\text{purity of technical material} * 1000}{\text{Nominal test item solution concentration} * \text{Content of AI \%}}$$

[3]: **g/L referred to the test item** based on nominal test item solution concentration of 30 mg/mL, Density of test item of 1.119 g/mL; calculated as

$$\frac{\text{Final Conc} * \text{density} * 1000}{\text{Nominal test item solution concentration}}$$

Validation - Results and discussions

Specificity

The specificity for all impurities was assessed with HPLC-MS/MS technique. Solutions for every standard and test item were analysed with HPLC-MS/MS instrument for specificity analysis. Solutions were prepared by diluting the sample in Acetonitrile.

Linearity

The linear range of the detector response was determined for each impurity by injecting the standard solutions described above. Range of calibration parameters and correlation coefficient of the curves are reported below (y = 100,795.5405x - 15.2939):

Parameters of the linearity curve for Simazine

Lower point	Higher point	a parameter (y = ax +b)	b parameter (y = ax + b)	R^2
0.0013 mg/mL (0.0922 g/Kg tech material) [1]	0.1303 mg/mL (9.2192 g/Kg tech material) [1]	100795.54	-15.29	0.9996

[1] based on nominal test item solution concentration of 30 mg/mL, %purity of technical material of 95%, Content of AI % of 44.76%.

Precision (Repeatability)

Independent test item solutions were prepared as described in above and analysed with the HPLC-UV method above. The results were summarized in below.

Summary of Content for Simazine

Sample No.	Area	Conc.	Test item weight	Volume	Dilution factor	Content		
-	[mAU*s]	[mg/mL]	[g]	[mL]	-	% w/w	g/Kg referred to technical material[1]	g/L to referred to test Item[2]
22363-sim 1	383.107	0.0039	0.2522	10	1	0.0157	0.333	0.175
22363-sim 2	418.868	0.0042	0.2781	10	1	0.0155	0.329	0.173

22363-sim 3	527.21	0.0053	0.3351	10	1	0.0161	0.341	0.180
22363-sim 4	482.914	0.0049	0.3117	10	1	0.0159	0.337	0.177
22363-sim 5	578.068	0.0058	0.3665	10	1	0.0161	0.341	0.180
MEDIA						**0.0158**	**0.336**	**0.177**
DEV.ST						**0.0002**	**0.005**	**0.0028**
% RSD						**1.58**	-	-
% RSDr						**5.00**	-	-
Hr						**0.32**	-	-

[1]: g/Kg referred to technical material based on %purity of technical material of 95%, Content of AI % of 44.76%.

[2]: g/L referred to the test item based on Density of test item of 1.119 g/mL

Accuracy

To determine the accuracy of the analytical methods, recovery experiments, were performed by fortification of test item. Recovery was prepared at two levels, LOQ and an appropriate second level set between the higher concentration found in test item and 80 per cent of the concentration of the last point in the calibration curve.

As acceptability criteria, a Mean Recovery of 80-120 % was adopted.

Accuracy results for Simazine

Recovery solution Code	**Area**	**C_F**	**C_U**	**C_A**	**Recovery**	**Mean recovery**
[-]	**[mAU*s]**	**[mg/mL]**	**[mg/mL]**	**[mg/mL]**	**[%]**	**[%]**
REC LOQ 1	829.3	0.0084	0.0051	0.0033	99.2	**103.1**
REC LOQ 2	820.3	0.0083	0.0049	0.0033	105.3	
REC LOQ 3	821.4	0.0083	0.0048	0.0033	107.0	
REC LOQ 4	859.7	0.0087	0.0052	0.0033	106.4	
REC LOQ 5	793.0	0.0080	0.0048	0.0033	97.8	
REC MAX 1	8836.8	0.0878	0.0051	0.0931	88.9	**90.7**
REC MAX 2	9151.7	0.0909	0.0048	0.0931	92.6	

Limit of quantification (LOQ)

The limit of quantification (LOQ) according to SANCO/3030/99 rev. 5 is defined as the lowest concentration tested at which an acceptable mean recovery with an acceptable RSD is obtained.

LOQ was tested by injecting five distinct recovery tests.

Summary of precision of Simazine at LOQ

Test item concentration	**Recovery level at LOQ**		**Mean recovery**	**Standard dev.**	**RSD**	**RSDr**	**Horrat**
[mg/mL]	[mg/mL]	[% w/w]	[%]	[-]	[%]	[%]	[-]
30	0.0033	0.011	103.1	4.30	4.17	5.29	0.79

Conclusion

A HPLC-DAD method was validated for determination of relevant impurities. Method validation included linearity, non-analyte interference, precision, accuracy and specificity. All measured parameters meet the criteria given in SANCO/3030/99 rev.5, 22 March 2019.

A 2.1.1.2 Description of analytical methods used in ecotoxicological studies

A 2.1.1.2.1 HPLC-MS/MS (in soil)

A 2.1.1.2.1.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/01
Report	Predatory mite *Hypoaspis (Geolaelaps) aculeifer* reproduction test in soil with H-01-2022 (terbuthylazine 500 g/L) - Analytical Phase, Report No.: 1015.1H.SAG23 / 23133-01R, Mautino G., 2023
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for terbuthylazine in soil was fully validated during the study according to the guideline SANTE/2020/12830 rev.2, of 14 February 2023, by calibration (linearity), selectivity, specificity, blank samples analysis, recoveries, accuracy, repeatability, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).
Terbuthylazine was extracted from soil samples with acidified acetonitrile, after an addition of an opportune amount of water. After QuEChERS salts addition, the acetonitrile phase was separated from the aqueous phase. The final analysis was performed in positive ionisation mode by High Performance Liquid Chromatography, tandem Mass Spectrometry (LC-MS/MS).
Two transitions m/z 230 > 174 and m/z 230 > 132 were acquired: the first transition (target) for quantification purpose, the second transition (qualifier) for confirmation purpose.

Chromatographic conditions

- LC System: HPLC Series 1200 Agilent
- MS/MS detector System: Triple Quadrupole API 3200 AB Sciex with TurboV Source
- Analytical Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm pore size, Agilent
- Mobile phases: Solvent A: Water (milliQ), 0.1% formic acid
  Solvent B: Methanol (UHPLC-MS), 0.1% formic acid
- Pump Gradient: 0 min: A 40 % - B 60 %
  5 min: A 40 % - B 60 %
- Flow rate: 0.3 mL/min
- Column Temperature 35 °C
- Injection volume: 2 µL
- Retention time: Terbuthylazine: ~2.2 minutes
- Mass Detector: Ionisation mode: ESI positive (MRM)

Temperature (TEM): 500 °C
Curtain gas (CUR): 20 psi
Collision gas (CAD): 5 psi
Ion Spray Voltage (IS): 5500 V
Gas 1: 45 psi
Gas 2: 45 psi
DP: 45
EP: 5.00

- Transitions: TBA-1_T (230/174) CE 22, CXP 2.5, dwell time 200 msec
  TBA-2_Q (230/132): CE 33, CXP 2.5, dwell time 200 msec

Analytical procedure for soil samples analysis
The sample was let to warm up to room temperature.
The homogenised sample (5 ± 0.1 g) was weighted into a 50 mL centrifuge tube: recovery samples were fortified at this point.
Then 8 mL of demineralised water were added and the sample was shaken for a few seconds using a vortex shaker to homogenise.
Then 10 mL of acetonitrile with 1% formic acid were added, the sample was manually shaken for two minutes and then shaken on a horizontal shaker for 20 minutes. After this step, the content of a sachet of QuEChERS was added to the sample.
The tube was shaken vigorously by hand for 2 minutes and then shaken on a horizontal shaker for 20 minutes. The sample was then centrifuged at 4000 rpm for five minutes.
1 mL of the supernatant layer was transferred into a 10 mL volumetric flask and made up to volume with acetonitrile (final extract volume 100 mL).
The final extract was filtered with PTFE filter, porosity 0.20 µm and analysed by LC-MS/MS method.
Quantification was conducted using an external standard calibration curve obtained by linear regression of matrix matched calibration standards injected throughout the run in the range 0.5 – 50 ng/mL.
Sample extracts with analyte concentration exceeding 40 ng/mL were opportunely diluted with the untreated blank extract in order to fall within the ± 20 % of the calibration range.

Validation

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

Linearity and instrumental precision
The linearity range for terbuthylazine was found between 0.5 - 50 ng/mL corresponding to 0.012 to 1.22 mg/kg of terbuthylazine in dry soil sample. The correlation coefficient of the weighed linear (1/x) multipoint external matrix matched standard calibration curves was found ≥ 0.999 for both ion transitions in all the analytical sequences performed.
The linearity range comprised the concentration range from 30 % of the LOQ to at least 20 % above the highest measured concentration.

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

$$di = yi - yyi$$

where:
yi is the measured value i;
yyi is the estimated value which corresponds to yi and is derived from the calibration function.

The regression residuals were presented in residual plots and visual inspections were applied to decide if di were randomly distributed and hence linear calibration is demonstrated: no trend was visible by plot-

ting the residuals vs the concentration.

Blank and selectivity

Two independent analyses of the blank sample were performed: no significant interference exceeding 30 % of the limit of quantification were found at the retention time of terbuthylazine for both the monitored transitions.

Therefore, terbuthylazine can be regarded as not detectable in untreated soil sample used in fortification trials (< 30 % of LOQ).

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

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

Matrix effects

To check possible signal enhancement or suppression effect in the LC-MS/MS analysis, the control sample extract fortified to achieve the nominal concentration of terbuthylazine at 2 ng/mL (nearest to the nominal concentration for the LOQ level) was compared to terbuthylazine in solvent at the same concentration. The results are summarized in the table below.

Transition	**Matrix response over solvent response %**	**Matrix effects %**
230/174 (Target)	88	-12
230/132 (Qualifier)	85	-15

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

Recovery

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

The LOQ level was set at terbuthylazine concentration of 0.05 mg/kg dry weight (lower than the minimum found terbuthylazine content in samples, while the second level was at 1402 mg/kg dry weight (higher than the maximum expected concentration in the samples), in order to cover with the method validation all the range of terbuthylazine concentrations in the analytical samples; five replicated analyses were carried out for each fortification level.

The background content in the control sample used for fortification experiments was not detectable. In these recovery samples the terbuthylazine content was determined as reported in table below.

Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	88.6	89.6	12.9	87.6 ± 9.3
	79.4			
	93.2			
	107.3			
	79.4			
1402 (II level)	84.1	85.6	2.8	
	87.1			
	82.1			
	87.1			
	87.7			

DW = Dry Weight

For each fortification level the mean recovery was in the range 70 – 120 % and the precision (RSD, rela-

tive standard deviation) ≤ 20 %, in compliance with the requirements of guideline SANTE/2020/12830 rev.2.

Accuracy
The accuracy of the analysis method for terbuthylazine in soil, defined as mean recovery ± relative standard deviation, is 87.6 ± 9.3.

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

Fortification level (mg/kg DW)	**RSD % (n = 5)**	**Overall RSD % (n = 10)**
0.05 (LOQ)	12.9	9.3
1402 (II level)	2.8	

Limit of quantification and limit of detection
The limit of quantification (LOQ) is defined as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained.
The LOQ for terbuthylazine in soil was assessed in this study at 0.05 mg/kg (referred to dry soil).

The limit of detection is the lowest amount that can be detected but not necessarily quantitated as an exact value.
For the analysis of terbuthylazine in soil the LOD is 0.012 mg/kg (referred to dry soil). This value, calculated from the terbuthylazine concentration corresponding to the lowest calibration point, is below 30 % of LOQ.

Confirmation
The confirmation of the analyte identity is simultaneous to the primary detection by the acquisition of the additional transition.
The recovery data and the precision data for the additional transition are reported in table below.

Fortification level (mg/kg DW)	**Recoveries (%)**	**Mean Recovery (%)**	**RSD (%)**	**Overall mean recovery ± RSD %**
0.05 (LOQ)	91.3	91.9	13	88.7 ± 9.9
	79.3			
	96.0			
	109.8			
	83.3			
1402 (II level)	81.9	85.5	2.8	
	86.2			
	84.2			
	87.7			
	87.3			

DW = Dry Weight

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

Stability of final extracts and reference item solutions

During the analytical sequences the injection of intermediate standard solutions and QC samples (recoveries) were done to check the calibration, the accuracy of the method and the samples stability during the course of the analysis.
The final extracts were analysed within 24 hours form extraction. Moreover, the stability of the extracts during the analysis was proven by the acceptability of recoveries performed concurrently with the samples analysis.
The terbuthylazine reference item stock solution was proven to be stable for 3 months (93 days) after preparation at ≤ - 18 °C in the dark: the means from at least 5 replicate measurements for a fresh solution compared to a stored one (at ≤ - 18 °C in the dark) did not differ by more than 10%, according to SANTE/2020/12830, Rev.2.

Conclusion

The data presented above confirm that the validated analytical method provides a specific, reliable, accurate and precise procedure for the determination of terbuthylazine active ingredient in soil samples in the range 0.05 – 1402 mg/kg DW. The method was fully validated according to SANTE/2020/12830, Rev.2.

A 2.1.1.2.2 HPLC with DAD and PC detection (in soil)

A 2.1.1.2.2.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/02 (filed as KCP 10.5/01)
Report	SOIL MICROORGANISMS: NITROGEN TRANSFORMATION TEST OF H-01-2022, Report No.: AG-G1155, KIRAN YADAV C., 2023
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The analytical method for the determination of active ingredient content in dose solution to establish the stability and homogeneity was validated by establishing specificity, matrix effect, linearity, LOD, LOQ, and recovery.
The determination was accomplished by the high performance liquid chromatography (HPLC) with DAD and PC detection.

Chromatographic Conditions

- Instrument: High Performance Liquid Chromatograph equipped with DAD and PC based data system.
- Column: Inertsil ODS-3V, 250 mm long, 4.6 mm i.d., 5 µm particle size.
- Mobile Phase: 0.1% Orthophosphoric acid in Milli-Q Water: Acetonitrile, 30 : 70 (% v/v)
- Detector Wavelength: 220 nm
- Flow Rate: 1.0 mL/min
- Injection Volume: 10 µL
- Column Temperature: 30°C
- Run time: 15 min

All the parameters were maintained constant throughout the analysis.
After every 3 to 9 sample injections and after the last sample injection, an injection of the calibration (standard) solution was made.
Terbuthylazine peak in the sample and in the standard was identified by comparing the retention times with those obtained through separate injections of solutions.
Peak area of Terbuthylazine for each injection was recorded.
The calibration (standard) solution peak area (injections before and after the sample injections) was averaged and used for calculating the percent active ingredient (a.i.) of Terbuthylazine in the samples.

Validation

Specificity
The specificity of the method was established by injecting the individual solutions of blank, working standard and test item into HPLC-PDA. There was no interference observed at the retention time of the analyte Terbuthylazine, confirmed the specificity of analytical method.

Matrix effect
The matrix effects on the method were found to be -12.71 % for Terbuthylazine which is less than 20%.

Linearity
Preparation of reference standard (Terbuthylazine) stock solution:
Accurately 0.0100 g of Terbuthylazine reference standard (purity 98.4 %m/m) was weighed into a 10 mL volumetric flask, dissolved in about 4 mL of Acetonitrile by sonicating for 2 minutes. After equilibrating to room temperature solution was diluted up to the mark with Acetonitrile.
Further, aliquots of this solution were diluted using Acetonitrile as detailed below.

Conc. of stock solution (µg/mL)	Further Dilution			
	Aliquot volume added (mL)	Volume of made up (mL)	Conc. Reference standard solution (µg/mL)	Solution ID
984	0.10	10	9.84	DLC-1
	0.15	10	14.76	DLC-2
	0.20	10	19.68	DLC-3
	1.0	10	98.40	DLC-4
	3.0	10	295.20	DLC-5

These working standard solutions were injected in triplicate into HPLC-PDA. A graph of detector response (peak area) versus concentration was plotted and linear regression coefficient (r) was established.
The instrument response was found to be linear in the concentration range of 9.84 µg/mL to 295.20 µg/mL for Terbuthylazine with r = 0.9984.

Recovery and Repeatability
To demonstrate recovery and repeatability of the method, Milli-Q water was fortified with test item at two appropriate levels (LOQ and 10 times LOQ) in five replications.
Low dose: About 0.12 g of test item was weighed in five replicates into separate 250 mL volumetric flasks, The contents were dissolved and diluted up to the mark using Milli-Q water. Further an aliquot of 5 mL of the stock solution was transferred into separate 50 mL volumetric flasks, dissolved and diluted up to the mark using Acetonitrile.
High dose: About 0.12 g of test item was weighed in five replicates into separate 25 mL volumetric flasks, The contents were dissolved and diluted up to the mark using Milli-Q water. Further an aliquot of 0.5 mL of the stock solution was transferred into separate 50 mL volumetric flasks, dissolved and diluted up to the mark using acetonitrile.
These diluted samples were analysed for a.i. content using HPLC-DAD.
From the mean ± s.d. of percent recovery, the mean value was taken as accuracy of the method at that fortification level.

Recovery of the method Terbuthylazine, as mean % recovery was 102.94 ± 0.67 and 102.43 ± 0.70 respectively for low dose and high dose respectively. The method was considered acceptable, since the obtained % recovery for Terbuthylazine was in the range of 70 to 120%.

Stability of Test Item Stock Solution
Preparation of reference standard (Terbuthylazine) stock solution:
Accurately 0.0100 g of Terbuthylazine reference standard (purity 98.4 %m/m) was weighed into a 10 mL volumetric flask, dissolved in about 4 mL of Acetonitrile by sonicating for 2 minutes. After equilibrating to room temperature solution was diluted up to the mark with Acetonitrile.
Further an aliquot of 0.2 mL of the above solution was transferred into a 10 mL volumetric flask, dissolved and diluted up to the mark using acetonitrile. This diluted solution was used as working standard solution for stability test and homogeneity test. (Conc: 20.0 µg/mL, without purity correction)
The first replication solution from recovery test, low dose equivalent to dosing concentration was stored at room temperature for a period of 24 hours.
After 24 hours, an aliquot of 5.0 mL from the stored solution was transferred into a 50 mL volumetric flask, dissolved and diluted up to the mark using Acetonitrile.
This diluted solution was analysed in duplicate injections using the validated analytical method to check the stability of the dose solution.

The test item stock solution was found to be stable as the analysed concentration after 24 hours is 99.56% of initial concentration.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). For the analysis of terbuthylazine in soil the LOD is 9.84 µg/mL.

The lowest fortified concentration used during accuracy test is considered as the Limit of quantification (LOQ). The LOQ for terbuthylazine in soil was assessed in this study at 233.97 µg/mL.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in soil.

A 2.1.1.2.3 LC-MS/MS (in reconstituted water)

A 2.1.1.2.3.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/03 (filed as KCP 10.2.1.2/01)
Report	H-01-2022: *Daphnia magna*, ACUTE IMMOBILIZATION TEST, Report No.: AG-G1146, LIKITH N.G., 2023
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples

Method validation and stability test was carried out at the following test item concentrations in the matrix- reconstituted water.

- Low dose – 101.00 µg/L (46.7125 µg Terbuthylazine/L)
- High dose – 101.00 mg/L (46.7125 mg Terbuthylazine/L)

Preparation of Test Sample

Accurately 0.0101 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up with reconstituted water and sonicated for 2 minutes. This results in a solution of concentration of 101.00 mg/L and was labelled as 'High dose'.

An aliquot of 0.1 mL of 'High dose' was transferred into 100 mL volumetric flask and volume was made up with reconstituted water, This results in a solution of concentration of 101.00 µg/L and was labelled as 'Low dose.'

Preparation of Working Standard Solutions

Working standard solution of concentration, approximately 0.023 µg/mL Terbuthylazine was prepared.

Standard stock solution of Terbuthylazine: Approximately 2.5 mg of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.

Working Standard Solution: Aliquots of 0.5 mL of the standard stock solution was transferred to 50 mL volumetric flask and the volume made up to the mark with diluent. Further 0.23 mL of the solution was transferred to the 10 mL volumetric flask and the volume was made up to the mark with diluent. This result in the working standard solution of approximately 0.023 µg/mL of Terbuthylazine.

Prepared two working standard solutions (starting from different weighing) and use one for system suitability test and the other for sample analysis (as bracketing calibration standards).

Preparation of Test Sample Solutions

Samples from the test samples in five composite replications for method validation was taken diluted using diluent such a way to get the final concentration of approximately 0.023 µg/mL of Terbuthylazine.

Low Dose (101.00 µg/L): Diluted 5 mL of the sample to 10 mL volumetric flask and diluted with diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.

High Dose (101.00 mg/L): Diluted 1.1 mL of the sample to 50 mL volumetric flask and diluted using diluent. Further 0.23 mL of this solution was transferred to 10 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.

Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions

- Scan type: MRM
- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00L/min
- Heating gas flow: 10L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00 L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.
The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity
Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.0129 to 0.0352 µg/mL with a regression coefficient (r) of 0.9953. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
Matrix effects was performed by comparing the analyte response one individual standard prepared in

blank matrix was compared with one prepared in solvent. The obtained matrix effect was 6.16%.

Linearity

For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00252 g of Terbuthylazine (purity: 98.4%) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 99.187 µg/mL.

Further, an aliquot of 0.5 mL of the standard stock solution was transferred to separate 50 mL volumetric flask, then the volume was made up to mark with diluent and mixed well. This resulted in the DLC Stock solution of concentrations of 0.992 µg/mL.

For detector linearity, a series of five working standard solutions in the range of 0.0129 to 0.0352 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of standard stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)
DLC1	0.992	0.130	10	0.0129
DLC2		0.180	10	0.0179
DLC3		0.230	10	0.0228
DLC4		0.290	10	0.0288
DLC5		0.355	10	0.0352

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision

Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.

Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g) taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.1) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

The accuracy of the method, as mean percent recovery at 46.7125 µg Terbuthylazine/L and 46.7125 mg Terbuthylazine/L fortification levels were 99.493 and 96.302 with a precision (as % RSD) of 0.949 and 0.882, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Stability

Stability of test item in the matrix was performed at low dose and high dose level.

The test samples after sampling for accuracy/precision test were retained at room temperature till the completion of stability test.

Samples were taken for analysis at the following intervals:

- 0 hour (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
- At 24 hours (samples stored at room temperature)
- At 48 hours (samples stored at room temperature)

On each stability interval three composite samples (except on 0-hour analysis) for each dose group (low and high) were drawn/taken and analysed for test item concentration.
Stability of the test sample was considered acceptable as the recoveries are within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

The stability of the test item in the test sample was evaluated for nominal dose concentration at 46.7125 µg Terbuthylazine/L and 46.7125 mg Terbuthylazine/L fortification levels in the matrix. The concentrations of the test items in the dose formulations were determined after 48 hours for the dose formulation stored at room temperature condition.
The mean percent recovery in the test item concentrations in the dose formulation stored at room temperature after 24 hours were 98.655 and 98.346 with % RSD 2.418 and 0.953 at the dose concentration of 46.7125 µg Terbuthylazine/L and 46.7125 mg Terbuthylazine/L, respectively.
The mean percent recovery in the test item concentrations in the dose formulation stored at room temperature after 48 hours were 98.086 and 96.932 with % RSD 0.232 and 1.615 at the dose concentration of 46.7125 µg Terbuthylazine/L and 46.7125 mg Terbuthylazine/L, respectively.

Solution (Injection Medium) Stability
Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24hours and 48 hours.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours, 48 hours, 72 hours and 169 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.
The %difference in all cases were within the study plan acceptance criteria of ±10.0% of initial value indicating that the standard solution was stable up to 169 hours when stored at room temperature.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0129 to 0.0352 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e. 0.0129 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test were 101.00 µg/L. The LOQ was calculated by considering the active content in the test item. LOQ = 46.7125 µg Terbuthylazine/L.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in reconstituted water.

A 2.1.1.2.4 LC-MS/MS (in algal water)

A 2.1.1.2.4.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/04 (filed as KCP 10.2.1.3/01)
Report	H-01-2022: ALGA GROWTH INHIBITION TEST WITH *Raphidocelis subcapitata*, Report No.: AG-G1147, LIKITH N.G., 2023
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix- algal water.

- Low dose – 102.00 µg/L (47.1750 µg Terbuthylazine/L)
- High dose – 102.00 mg/L (47.1750 mg Terbuthylazine/L)

Preparation of Test Sample
Accurately 0.0102 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up with algal water and sonicated for 2 minutes. This results in a solution of concentration of 102.00 mg/L and was labelled as 'High dose'.
An aliquot of 0.1 mL of 'High dose' was transferred into 100 mL volumetric flask and volume was made up with algal water, This results in a solution of concentration of 102.00 µg/L and was labelled as 'Low dose.'

Preparation of Working Standard Solutions
Working standard solution of concentration, approximately 0.023 µg/mL Terbuthylazine was prepared.
Standard stock solution of Terbuthylazine: Approximately 2.5 mg of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.
Working Standard Solution: Aliquots of 0.5 mL of the standard stock solution was transferred to 50 mL volumetric flask and the volume made up to the mark with diluent. Further 0.23 mL of the solution was transferred to the 10 mL volumetric flask and the volume was made up to the mark with diluent. This result in the working standard solution of approximately 0.023 µg/mL of Terbuthylazine.
Prepared two working standard solutions (starting from different weighing) and use one for system suitability test and the other for sample analysis (as bracketing calibration standards).

Preparation of Test Sample Solutions
Samples from the test samples in five composite replications for method validation was taken diluted using diluent such a way to get the final concentration of approximately 0.023 µg/mL of Terbuthylazine.
Low Dose (102.00 µg/L): Diluted 5 mL of the sample to 10 mL volumetric flask and diluted with diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.

High Dose (102.00 mg/L): Diluted 1.1 mL of the sample to 50 mL volumetric flask and diluted using diluent. Further 0.23 mL of this solution was transferred to 10 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.
Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions

- Scan type: MRM
- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00 L/min
- Heating gas flow: 10 L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00 L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.
The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity
Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance

criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.0129 to 0.0352 µg/mL with a regression coefficient (r) of 0.9953. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low dose (at injection) and this was used as diluent, to prepare the matrix matched standard.
Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was -0.19%.

Linearity
For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00252 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 99.187 µg/mL.
Further, an aliquot of 0.5 mL of the standard stock solution was transferred to separate 50 mL volumetric flask, then the volume was made up to mark with diluent and mixed well. This resulted in the DLC Stock solution of concentrations of 0.992 µg/mL.
For detector linearity, a series of five working standard solutions in the range of 0.0129 to 0.0352 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of standard stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)
DLC1	0.992	0.130	10	0.0129
DLC2		0.180	10	0.0179
DLC3		0.230	10	0.0228
DLC4		0.290	10	0.0288
DLC5		0.355	10	0.0352

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision
Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g)taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.1) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.
The accuracy of the method, as mean percent recovery at 47.1750 µg Terbuthylazine/L and 47.1750 mg Terbuthylazine/L fortification levels were 97.037 and 95.069 with a precision (as % RSD) of 2.277 and 0.709, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Solution (Injection Medium) Stability
Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24hours and 48 hours.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours, 48 hours, 72 hours and 169 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions working standard solution stored at ambient temperature after 24 hours, 48 hours, 72 hours and 169 hours and sample solutions corresponding to dose formulation concentrations stored at ambient temperature after 24 hours and 48 hours.

The % difference for the standard solutions while stored at ambient temperature for 169 hours was -1.00%.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours and 48 hours.

The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.
The %difference in all cases were within the study plan acceptance criteria of ±10.0% of initial value indicating that the standard solution was stable up to 169 hours when stored at room temperature.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0129 to 0.0352 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e. 0.0129 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test was 102.00 µg/L. The LOQ was calculated by considering the active content in the test item. LOQ = 47.1750 µg Terbuthylazine/L.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix - algal water.

A 2.1.1.2.5 LC-MS/MS (in Lemna water)

A 2.1.1.2.5.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/05 (filed as KCP 10.2.1.4/01)
Report	H-01-2022: *LEMNA* GROWTH INHIBITION TEST, Report No.: AG-G1148, LIKITH N.G., 2023
Guideline(s):	SANTE/2020/12830, rev.1
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix- Lemna water.

- Low dose – 102.00 µg/L (47.1750 µg Terbuthylazine/L)
- High dose – 102.00 mg/L (47.1750 mg Terbuthylazine/L)

Preparation of Test Sample
Accurately 0.0102 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up with algal water and sonicated for 2 minutes. This results in a solution of concentration of 102.00 mg/L and was labelled as 'High dose'.
An aliquot of 0.1 mL of 'High dose' was transferred into 100 mL volumetric flask and volume was made up with algal water, This results in a solution of concentration of 102.00 µg/L and was labelled as 'Low dose.'

Preparation of Working Standard Solutions
Working standard solution of concentration, approximately 0.023 µg/mL Terbuthylazine was prepared.
Standard stock solution of Terbuthylazine: Approximately 2.5 mg of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.
Working Standard Solution: Aliquots of 0.5 mL of the standard stock solution was transferred to 50 mL volumetric flask and the volume made up to the mark with diluent. Further 0.23 mL of the solution was transferred to the 10 mL volumetric flask and the volume was made up to the mark with diluent. This result in the working standard solution of approximately 0.023 µg/mL of Terbuthylazine.

Prepared two working standard solutions (starting from different weighing) and use one for system suitability test and the other for sample analysis (as bracketing calibration standards).

Preparation of Test Sample Solutions

Samples from the test samples in five composite replications for method validation was taken diluted using diluent such a way to get the final concentration of approximately 0.023 µg/mL of Terbuthylazine.

Low Dose (102.00 µg/L): Diluted 5 mL of the sample to 10 mL volumetric flask and diluted with diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.

High Dose (102.00 mg/L): Diluted 1.1 mL of the sample to 50 mL volumetric flask and diluted using diluent. Further 0.23 mL of this solution was transferred to 10 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 0.023 µg/mL and injected to LC-MS/MS.

Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions

- Scan type: MRM
- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00 L/min
- Heating gas flow: 10 L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00 L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.

The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.

Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).

The peak area of analyte for each injection was recorded.

The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity

Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.

Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.

Detector response was linear in the range of 0.0129 to 0.0352 µg/mL with a regression coefficient (r) of 0.9953. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect

Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.

The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low dose (at injection) and this was used as diluent, to prepare the matrix matched standard.

Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was 0.35%.

Linearity

For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00252 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 99.187 µg/mL.

Further, an aliquot of 0.5 mL of the standard stock solution was transferred to separate 50 mL volumetric flask, then the volume was made up to mark with diluent and mixed well. This resulted in the DLC Stock solution of concentrations of 0.992 µg/mL.

For detector linearity, a series of five working standard solutions in the range of 0.0129 to 0.0352 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of standard stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)
DLC1	0.992	0.130	10	0.0129
DLC2		0.180	10	0.0179
DLC3		0.230	10	0.0228
DLC4		0.290	10	0.0288
DLC5		0.355	10	0.0352

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision

Test item concentrations in the test samples at two levels (low and high dose) were analysed in five com-

posite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g)taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.1) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.
The accuracy of the method, as mean percent recovery at 47.1750 µg Terbuthylazine/L and 47.1750 mg Terbuthylazine/L fortification levels were 97.500 and 96.416 with a precision (as % RSD) of 0.883 and 0.434, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Solution (Injection Medium) Stability
Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24hours, 48 hours, 72 hours and 169 hours.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours, 48 hours, 72 hours and 169 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions (working standard solution, and sample solutions corresponding to dose formulation concentrations) stored at ambient temperature for 48 and 169 hours.
The % difference for the standard solutions while stored at ambient temperature for 24 hours was -0.10%.
The % difference for the standard solutions while stored at ambient temperature for 48 hours was -0.48%.
The % difference for the standard solutions while stored at ambient temperature for 72 hours was -0.69%.
The % difference for the standard solutions while stored at ambient temperature for 169 hours was -1.00%.

The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 169 hours.

The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.

The %difference in all cases were within the study plan acceptance criteria of ±10.0% of initial value indicating that the standard solution was stable up to 169 hours when stored at room temperature.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0129 to 0.0352 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e. 0.0129 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test was 102.00 µg/L. The LOQ was calculated by considering the active content in the test item. LOQ = 47.1750 µg Terbuthylazine/L.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.1. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix - Lemna water.

A 2.1.1.2.6 LC-MS/MS (in artificial soil)

A 2.1.1.2.6.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/06 (filed as KCP 10.4.1.1/01)
Report	H-01-2022: EARTHWORM REPRODUCTION TEST (*Eisenia fetida*), Report No.: AG-G1153, VISHALA N., 2023
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The solution stability of working standard and sample solutions under specified storage conditions was also assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations:
- Low dose – 0.010 mg test item/kg dw soil
- High dose – 1000.889 mg test item/kg dw soil

Preparation of Test Sample
Low dose - 0.010 mg test item/kg dw soil: Accurately 0.0101 g of test item was weighed into a 100 mL volumetric flask, then the volume was made up to the mark with deionized water and sonicated for 2minutes. This results in a solution with a concentration of approximately 101.00 mg/L and was labelled as 'Low dose stock1'.
An aliquot of 1mL of 'Low dose stock1' (approximately 101.00mg/L) was transferred to 100mL volumet-

ric flask and the volume was made upto the mark with deionized water. This results in a solution with a concentration of approximately 1.01 mg/L and was labelled as 'Low dose stock2.'

An aliquot of 1.0 mL of 'Low dose stock 2' (approximately 1.01 mg/L) was added to 100.0021g of artificial soil and 26 mL of deionized water was added and mixed homogenously. This will result in a test item of concentration of approximately 0.010 mg/kg dw soil and it was labelled as 'Low dose'.

High Dose-1000.889 mg test item/kg dw soil: Accurately 0.1001 g of test item was weighed and mixed with 27 mL of deionized water. Then it was added to 100.0110 g of artificial soil and mixed homogenously. It was labelled as 'High dose'.

Preparation of Working Standard Solutions

Working standard solution of concentration, approximately 0.023 µg/mL Terbuthylazine was prepared.
Standard stock solution of Terbuthylazine: Approximately 2.5 mg of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.
Working Standard Solution: Aliquots of 0.1 mL of the stock was transferred to 100 mL volumetric flasks and made up to the mark using diluent. Further 1.15 mL of above solution was transferred to 50 mL volumetric flask and made up to the mark using diluent. This results in a working standard solution of approximately 0.0023 µg/mL.
Prepared two working standard solutions (starting from different weighing) and used one for system suitability test and the other for sample analysis.

Preparation of Test Sample using Dry Weight Soil

Samples from the fortified test medium in five replicates for method validation were taken and then extracted with extraction solvent and further diluted using diluent (Acetonitrile) to get the final concentration of approximately 0.0023 µg/mL of Terbuthylazine.
Low dose (0.010 mg test item/kg dw soil): Approximately 5 g of test samples were transferred into a 100 mL conical flask and 10 mL of extraction solvent was added. And the process was continued by shaking in a mechanical platform shaker at an approximate speed of 180 rpm for 20 minutes. The whole samples were transferred to 15 mL Tarson tube and centrifuged at an approximate speed of 3000 rpm at 25°C for 10 minutes. This results in a solution of approximately 0.0023 µg /mL of Terbuthylazine which was injected to LC-MS/MS.
High Dose (1000.889 mg test item/kg dw soil): Approximately 5 g of test samples was transferred into suitable container and 10 mL of extraction solvent was added, and the process was continued by shaking in a mechanical platform shaker at an approximate speed of 180 rpm for 20 minutes. The whole samples were transferred to 15mL Tarson tube and centrifuged at an approximate speed of 3000 rpm at 25°C for 10 minutes and 0.22 mL of supernatant was transferred in 50 mL volumetric flasks and made up to the mark using diluent. Further 0.115 mL of above solution was transferred to 50 ml volumetric flask and made up to the mark using diluent.
This results in a solution of approximately 0.0023 µg /mL of Terbuthylazine which was injected to LC-MS/MS.
Similarly, one control sample was processed for both low and high dose.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions

- Scan type: MRM

- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00 L/min
- Heating gas flow: 10 L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00 L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections using external working standard solution.
Analyte peak in the sample were identified by comparing the retention time of the analyte peak in the standard with that of the analyte peak in the sample. Peak areas of Terbuthylazine for each injection were recorded.

Validation

Specificity
Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.00121 to 0.00353 µg/mL (The equivalent calibration range in mg/kg i.e. 0.00242, 0.00362, 0.00464, 0.00584 and 0.00706 mg/kg respectively.) with a regression coefficient (r) of 0.9976 (Quantifier) and 0.9978 (Qualifier). This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low dose (at injection) and this was used as diluent, to prepare the matrix matched standard.

Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix. The obtained matrix effect was 1.663 % (Quantifier) and 1.273 % (Qualifier).

Linearity

For detector linearity, a DLC stock solution was prepared by transferring an accurate quantity of 0.00256 g of reference standard (Purity: 98.4 %) into a 25 mL volumetric flask, dissolved and made up to the mark with diluent (Acetonitrile) and mixed well to get a stock solution of 100.762 µg/mL.
Further an aliquot of 0.1 mL was diluted to 100 mL of volumetric flask. It results in a solution of concentration 0.1008 mg/L which was labelled as DLC Stock.
For detector linearity, a series of five working standard solutions in the range of 0.00121 to 0.00353 µg/mL of analyte were prepared by diluting a known volume (DLC Stock) with diluent as given below.

DLC Stock conc.	Aliquot of DLC stock taken (mL)	Volume made up to (mL)	Concentration of diluted solution (µg/mL)
0.1008 µg/mL	0.12	10	0.0121
	0.18	10	0.0181
	0.23	10	0.0232
	0.29	10	0.0292
	0.35	10	0.0353

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision

Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.
The accuracy of the method, as mean percent recovery at 0.00463 mg Terbuthylazine/kg dw and 462.911 mg Terbuthylazine/kg dw soil fortification levels were 105.098 and 101.883 with a precision (as % RSD) of 0.750 and 0.624, respectively for Terbuthylazine Quantifier lower and higher fortification level.
The accuracy of the method, as mean percent recovery 0.00463 mg Terbuthylazine/kg dw and 462.911 mg Terbuthylazine/kg dw soil fortification levels were 104.795 and 102.561 with a precision (as % RSD) of 0.396 and 0.608, respectively for Terbuthylazine Qualifier lower and higher fortification level.
The obtained results met the acceptance criteria of recoveries to be within
70% to 120% and precision as % RSD to be < 20.0%.

Solution (Injection Medium) Stability
Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours.
The extracted sample solutions were considered stable as the obtained recoveries were within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours.
The means from at least 5 replicate measurements for each of the two solutions were within 10%.

The solution stability was evaluated for the analytical solutions (working standard solution, and sample solutions corresponding to dose formulation concentrations) stored at ambient temperature after 24 hours.

The % difference for the standard solutions while stored at ambient temperature after 24 hours was 0.550%
The %difference in all the cases was within the study plan acceptance criteria of ± 10.0% of indicating that the standard solution was stable up to 24 hours when stored at room temperature.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after 24 hours.
The processed sample solutions were considered stable, the obtained recoveries were within 70-120%.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.00121 to 0.00353 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate. The lowest calibration standard used in the linearity and range test i.e., 0.00121 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test was 0.010 mg/L.
The LOQ was calculated by considering the active content in the test item. LOQ = 0.00463 mg a.i./kg dw soil.

Conclusion

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

A 2.1.1.2.7 LC-MS/MS (in Smart and Barko Medium (water samples), sediment and sediment pore water)

A 2.1.1.2.7.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/07 (filed as KCP 10.2.1.4/02)
Report	H-01-2022: WATER SEDIMENT *MYRIOPHYLLUM SPICATUM* TOXICITY TEST, Report No.: AG-G1158, LIKITH N.G., 2023

Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix:
Smart and Barko Medium (water samples)
- Low dose – 0.0218 mg/L
- High dose – 109.00 mg/L

Sediment
- Low dose – 0.0211 mg/L
- High dose – 100.101 mg/L

Sediment pore water.
- Low dose – 0.0224 mg/L
- High dose – 103.00 mg/L

Preparation of Test Sample (Smart and Barko Medium)
Accurately 0.01090 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up with matrix and sonicated for 5 minutes. This results in a solution of concentration of 109.00 mg/L and was labelled as ‘High dose'.
An aliquot of 0.5 mL of ‘High dose’ (109.00 mg/L) was transferred into 50 mL volumetric flask and diluted using matrix and mixed well. This results in a solution of concentration of 1.090 mg/L and was labelled as ‘Low dose stock.’
An aliquot of 2.0 mL of ‘Low dose stock’ (1.090 mg/L) was transferred into 100 mL volumetric flask and diluted using matrix and mixed well. This results in a solution of concentration of 0.0218 mg/L and was labelled as ‘Low dose.’

Preparation of Test Sample (Sediment)
Accurately 0.50313 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up to the matrix with deionized water and sonicated for 10 minutes. This results in a solution of concentration of 5031.3 mg/L and was labelled as ‘High dose stock'.
An aliquot of 1.0 mL of ‘High dose stock’ (5031.3 mg/L) was added to 50.262 g of test substrate and mixed homogenously for 5 minutes. This resulted in a test item of concentration of approximately 101.101 mg/g and was labelled as ‘High dose.’

Accurately 0.01065 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up to the matrix with deionized water and sonicated for 10 minutes. This results in a solution of concentration of 106.5 mg/L and was labelled as ‘Low dose stock-1'.
An aliquot of 1.0 mL of ‘Low dose stock-1’ (106.5 mg/L) was transferred into a 100 mL volumetric flask and diluted using matrix and mixed well. This results in a solution of concentration of 1.065 mg/L and was labelled as ‘Low dose stock-2'.
An aliquot of 1.0 mL of ‘Low dose stock-2’ (1.06. mg/L) was added to 50.301 g of test substrate and mixed homogenously for 5 minutes. This resulted in a test item of concentration of approximately 0.0212 mg/g and was labelled as ‘Low dose.’

Preparation of Test Sample (Sediment pore water)
Accurately 0.1030 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up to the matrix with deionized water and sonicated for 10 minutes. This results in a solution of concentration of 1030.00 mg/L and was labelled as 'High dose stock'.
An aliquot of 2.5 mL of 'High dose stock' (1030.0 mg/L) was transferred into 25 mL volumetric flask and diluted using matrix and mixed well. This resulted in a solution of concentration of 103.00 mg/g and was labelled as 'High dose.'

Accurately 0.0112 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up to the matrix with deionized water and sonicated for 10 minutes. This results in a solution of concentration of 112.00 mg/L and was labelled as 'Low dose stock'.
An aliquot of 100 mL of 'Low dose stock' (112.00 mg/L) was transferred into 50 mL volumetric flask and diluted using matrix and mixed well. This results in a solution of concentration of 0.0224 mg/L and was labelled as 'Low dose.'

Preparation of Test Sample Solutions
Barko Medium (Water samples)
Samples from the test samples in five composite replications for method validation were taken and diluted using diluent in such a way to get the final concentrations approximately ranging from 0.001 µg/mL to 0.1 µg/mL.
Low Dose (0.0218 mg/L): Diluted 5 mL of sample to 10 mL with diluent and injected to LC-MS/MS.
High Dose (109.000 mg/L): Diluted 1.1 mL of sample to 50 mL with diluent and further 0.25 mL was transferred to a 50 mL volumetric flask and made up to the mark using diluent and injected to LC-MS/MS.

While diluting the test sample % content of analyte (46.25%) was considered.
Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Sediment
Samples from the test sample in five replications for method validation and three replicates for A.I samples were taken and extracted using acetonitrile and further diluted using diluent in such a way to get the final concentrations ranging from 0.001 µg/mL to 0.1 µg/mL.
Low dose (0.0212 mg/g): Approximately 5 g of test samples was transferred into suitable container and 10 mL of acetonitrile was added. The process was continued by shaking in a mechanical platform shaker at room temperature at an approximate speed of 180 rpm for 20 minutes. The whole sample was centrifuged at an approximate speed of 3000 rpm at 25°C for 10 minutes, filtered and injected to LC-MS/MS.
High Dose (100.101 mg/g): Approximately 5 g of test samples was transferred into suitable container and 10 mL of acetonitrile was added, and the process was continued by shaking in a mechanical platform shaker at room temperature at an approximate speed of 180 rpm for 20 minutes The whole sample was centrifuged at an approximate speed of 3000 rpm at 25°C for 10 minutes, and 0.23 mL of supernatant was transferred into a 50 mL volumetric flask and made up to the mark with diluent. Further an aliquot of 2.5mL was transferred into a 50 mL volumetric flask and made up to the mark with diluent and injected to LC-MS/MS.
While diluting the test sample % content of analyte (46.25%) was considered.
Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Sediment pore water
Samples from the test samples in five composite replications for method validation was taken and diluted using diluent to get the final concentrations approximately ranging from 0.001 µg/mL to 0.1 µg/mL.
Low Dose (0.0224 mg/L): Diluted 5 mL of sample to 10 mL with diluent, filtered and injected to LC-MS/MS.
High Dose (103.000 mg/L): Diluted 1.1 mL of sample to 50 mL with diluent and further 0.25 mL was transferred to a 50 mL volumetric flask and made up to the mark using diluent and injected to LC-MS/MS.
While diluting the test sample % content of analyte (46.25%) was considered.

Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Column Temperature: 40°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (20:80% v/v)
- Flow rate: 1.0 mL/min
- Injection volume: 30 µL

MS Conditions

- Scan type: MRM
- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.100
  230.00/132.00

Source parameters

- Curtain gas: 25 psi
- CAD: 5 psi
- Ion spray voltage: 4500 V
- Ion source gas (GS1): 50 psi
- Ion source gas (GS2): 60 psi
- Temperature: 500°C
- De-clustering potential (DP): 40
- Entrance potential (EP): 8
- Collision energy (CE): 40

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.

Validation

Specificity
Diluent and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.001 to 0.098 µg/mL with a regression coefficient (r) of 0.9990. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low dose (at injection) and this was used as diluent, to prepare the matrix matched standard.
Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was:

- Smart and Barko medium: -2.75%
- Sediment: -11.42%
- Sediment pore water: 13.56%

Linearity
For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00248 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 97.613 µg/mL.
Further 0.5 mL of the above solution was transferred in to a 50 mL volumetric flask, then the volume was made up to mark with diluent. This resulted in the DLC Stock solution of concentrations of 0.976 µg/mL.
For detector linearity, a series of five working standard solutions in the range of 0.001 to 0.098 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of DLC stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)
DLC1	0.976	0.05	50	0.001
DLC2		0.15	50	0.003
DLC3		0.25	50	0.005
DLC4		3.75	50	0.073
DLC5		5.00	50	0.098

In addition, the regression residual (di) was measured as below:

$$di = Yi - Yyi$$

where:
Yi – measured value
Yyi – estimated value which corresponds to yi and I derived from the calibration function.
The regression residuals were represented in the residual plot.

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision
Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g)taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.

Smart and Barko medium

The accuracy of the method, as mean percent recovery at 0.0101 mg Terbuthylazine/L and 50.4125 mg Terbuthylazine/L fortification levels were 92.079 and 77.903 with a precision (as % RSD) of 3.261 and 4.141, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Sediment

The accuracy of the method, as mean percent recovery at 0.0098 mg Terbuthylazine/g and 46.2967 mg Terbuthylazine/g fortification levels were 109.184 and 92.661 with a precision (as % RSD) of 3.887 and 3.542, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Sediment pore water

The accuracy of the method, as mean percent recovery at 0.0104 mg Terbuthylazine/L and 47.6375 mg Terbuthylazine/L fortification levels were 109.615 and 102.287 with a precision (as % RSD) of 1.468 and 3.122, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Stability

Stability of test item in the matrix (Smart and Barko water) was performed at low dose and high dose level.

The test samples after sampling for accuracy/precision test was retained at room temperature till the completion of stability test.

Samples were taken for analysis at the following intervals:

- Day 0 (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
- Day-1 (samples stored at room temperature)
- Day-7 (samples stored at room temperature)
- Day-14 (samples stored at room temperature)

On each stability interval three composite samples (except on 0-hour analysis) for each dose group (low and high) were drawn/taken and analysed for test item concentration.

Stability of the test sample was considered acceptable as the recoveries were within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

Solution (Injection Medium) Stability

Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours and 48 hours

for Smart and Barko water and sediment and 121 hours for sediment pore water respectively.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours and 48 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions (standard solution) stored at ambient temperature for 24 and 48 hours and (sample solutions corresponding to dose formulation concentrations) stored at ambient temperature for 24 and 48 hours for Smart and Barko medium and sediment, 121 hours for sediment pore water.
The % difference for the lowest calibration standard solutions (CC1) while stored at ambient temperature for 24 hours was -5.139 %.
The % difference for the highest calibration standard solutions (CC5) while stored at ambient temperature for 24 hours was -7.000 %.
The % difference for the lowest calibration standard solutions (CC1) while stored at ambient temperature for 48 hours was -4.686%.
The % difference for the highest calibration standard solutions (CC5) while stored at ambient temperature for 48 hours was -2.647%.

The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.

The % difference in all cases were within the acceptance criteria of ± 10.0% of initial value indicating that the standard solution was stable up to 48 hours when stored at room temperature.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.001 to 0.098 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e 0.001 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test was 0.0218 mg/L (Smart and Barko medium), 0.0212 mg/g (Sediment) and 0.0224 mg/L (Sediment pore water).
The LOQ was calculated by considering the active content in the test item i.e. 0.0101 mg a.i./L (Smart and Barko medium), 0.0098 mg a.i./g (Sediment) and 0.0104 mg a.i./L (Sediment pore water).

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix: Smart and Barko Medium (water samples), sediment and sediment pore water.

A 2.1.1.2.8 LC-MS/MS (in Milli-Q water)

A 2.1.1.2.8.1 Method validation

Comments of Evaluator:	Method is accepted

Reference: KCP 5.1.2/08 (filed as KCP 10.2.1.4/02)

Report	H-01-2022: HONEYBEE (*Apis mellifera* L) LARVAL TOXICITY TEST, REPEATED EXPOSURE, Report No.: AG-G1149, GANGADHAR R. S., 2024
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix:
- Low dose – 0.051 µg ai/µL
- High dose – 12.025 µg ai/µL

Preparation of Test Sample
Accurately 2.6000 g of test item was weighed into a 100mL volumetric flask and the volume was made up to 100mL using matrix and sonicated for 5 minutes. This results in a solution of concentration of 12.025 µg ai/µL and was labelled as 'High dose'.
An aliquot of 0.42 mL of 'High dose' was transferred to 100mL volumetric flask and the volume was made up to 100mL using matrix. This results in a solution of concentration of 0.051 µg ai/µL and was labelled as 'Low dose.'

Preparation of Working Standard Solutions
Working standard solution of concentration, approximately 0.025 µg/mL Terbuthylazine was prepared.
Standard stock solution: Approximately 2.5 mg of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.
Working Standard Solution: Aliquots of 0.5 mL of the standard stock solution was transferred to 50 mL volumetric flask and the volume made up to the mark with diluent. Further 1.25 mL of the solution was transferred to the 50 mL volumetric flask and the volume was made up to the mark with diluent. This result in the working standard solution of approximately 0.025 µg/mL of Terbuthylazine.

Prepared two working standard solutions (starting from different weighing) and use one for system suitability test and the other for sample analysis (as bracketing calibration standards).

Preparation of Test Sample Solutions
Samples from the test samples in six composite replications (two replication each from top, middle and bottom layers) for method validation was taken diluted using diluent such a way to get the final concentration of approximately 0.025 µg/mL of Terbuthylazine.
Low Dose (0.051 µg ai/µL): An aliquot of 1 mL was transferred to 50 mL volumetric flask and made up to the mark using diluent. Further 1.25 of this solution was transferred to 50 mL volumetric flask and made up to the mark using the diluent to obtain the resulting solution of concentration of approximately 0.025 µg/mL and injected to LC-MS/MS.
High Dose (12.025 µg ai/µL): An aliquot of 0.1 mL was transferred to 50 mL volumetric flask and made up to the mark using diluent. Further 52µL of this solution was transferred to 50 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 0.025 µg/mL and injected to LC-MS/MS.

Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions

- Scan type: MRM
- Ionization mode: ESI +ve (polarity)
- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00 L/min
- Heating gas flow: 10 L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.
The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity
Diluent and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.

Detector response was linear in the range of 0.0118 to 0.0374 µg/mL with a regression coefficient (r) of 0.9998. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect

Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.

Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was -0.482%.

Linearity

For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00250 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 98.400 µg/mL.

Further, an aliquot of 0.5 mL of the standard stock solution was transferred to separate 50 mL volumetric flask, then the volume was made up to mark with diluent and mixed well. This resulted in the DLC Stock solution of concentrations of 0.984 µg/mL.

For detector linearity, a series of five working standard solutions in the range of 0.0120 to 0.0380 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	**Conc. of DLC Stock solution taken (µg/mL)**	**Volume of DLC stock solution taken (mL)**	**Volume made up to (mL)**	**Conc. of solution (µg/mL)**
DLC1	0.984	0.120	10	0.0118
DLC2		0.180	10	0.0177
DLC3		0.250	10	0.0246
DLC4		0.310	10	0.0305
DLC5		0.380	10	0.0374

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

In addition, the regression residual (di) was measured as below:

$$di = Yi - Yyi$$

where:
Yi – measured value
Yyi – estimated value which corresponds to yi and I derived from the calibration function.
The regression residuals were represented in the residual plot.

Accuracy and Precision

Test item concentrations in the test samples at two levels (low and high dose) were analysed in six replications (two replication each from top, middle and bottom layers) using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g)taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.

The accuracy of the method, as mean percent recovery at 0.051 µg ai/µL and 12.025 µg ai/µL fortification levels were 96.732 and 97.927 with a precision (as % RSD) of 1.047 and 0.608, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Stability

Stability of test item in the matrix was performed at low dose and high dose level.
The test samples after sampling for accuracy/precision test were retained at room temperature till the completion of stability test.
Samples were taken for analysis at the following intervals:
0 hour (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
At 4 hours (samples stored at room temperature)
On each stability interval three replications (one replication each from top, middle and bottom layers) for each dose group (low and high) was taken and analysed for test item concentration.
Stability of the test sample was considered acceptable as the recoveries are within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

Analytical Solution (Injection Medium) Stability

Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 169 hours.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 145 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions (working standard solution, and sample solutions corresponding to dose formulation concentrations) stored at ambient temperature for 169 hours.
The % difference for the standard solutions while stored at ambient temperature for 169 hours was 0.425%.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 169 hours.
The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.
The % difference in all cases were within the study plan acceptance criteria of ± 10.0% of initial value indicating that the sample solution was stable up to 169 hours when stored at room temperature.

Stability Test
The stability of the test item in the test sample was evaluated for nominal dose concentration at 0.051 µg ai/µL and 12.025 µg ai/µL fortification levels in the matrix. The concentrations of the test items in the dose formulations were determined after 4 hours for the dose formulation stored at room temperature condition.
The mean percent recovery in the test item concentrations in the dose formulation stored at room temperature after 4 hours were 96.732 and 98.226 with % RSD 1.170 and 0.395 at the dose concentration 0.051 µg ai/µL and 12.025 µg ai/µL, respectively.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0118 to 0.0374 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e 0.0118 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration used in the accuracy and precision was considered as LOQ. i.e. 0.051 µg ai/µL.

System Suitability and Standard Check
System suitability and standard check was performed on all the days of analysis and the obtained results met the acceptance criteria.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix: Milli-Q water.

A 2.1.1.2.9 LC-MS/MS (in 50% w/v Sucrose solution in Milli-Q water)

A 2.1.1.2.9.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/09 (filed as KCP 10.3.1.1.2/02)
Report	H-01-2022: CHRONIC ORAL TOXICITY TEST IN HONEYBEE (*Apis mellifera* L.), Report No.: AG-G1152, GANGADHAR R. S., 2024
Guideline(s):	SANTE/2020/12830, rev.2

Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The LC-MS/MS method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range, precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The stability of working standard and processed sample solution(s) under specified storage conditions was also be assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix:
- Low dose – 0.051 mg ai/kg diet
- High dose – 120.710 mg ai/kg diet

Preparation of Test Sample
Accurately 0.0261 g of test item was weighed into a pre-calibrated beaker and the final weight was made up to 100.0012 g using matrix and sonicated for 15 minutes. This results in a solution of concentration of 120.711 mg ai/kg and was labelled as 'High dose'.
An aliquot of 0.0421 g of 'High dose' was transferred into pre-calibrated beaker and the final weight was made up to 100.0010 g using matrix. This results in a solution of concentration of 0.051 mg ai/kg and was labelled as 'Low dose.'

Preparation of 50 % w/v sucrose solution in Milli-O water
Accurately, 125.0011 g of sucrose was weighed in a 250 mL beaker and transferred into a 500 mL pre-calibrated beaker containing 200 mL of Milli-Q water and it was stirred manually using glass rod. Then the volume was made up to 250 mL with Milli-Q water and it was placed on a magnetic stirrer for stirring for 15 minutes.

Preparation of Test Sample Solutions
Samples from the test samples in five composite replications for method validation was taken diluted using diluent such a way to get the final concentration of approximately 0.025 µg/mL of Terbuthylazine.
Low Dose (0.05 mg ai/kg): Diluted 5 mL of the sample to 10 mL volumetric flask and diluted with diluent to obtain the resulting solution of concentration of approximately 0.025 µg/mL and injected to LC-MS/MS.
High Dose (120 mg ai/kg): An aliquot of 1 mL of the sample was transferred to 50 mL volumetric flask and made up to the mark using diluent. Further 0.52 mL of this solution was transferred to 50 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 0.025 µg/mL and injected to LC-MS/MS.
Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions
- Instrument: LC-MS/MS mass spectrometer with liquid chromatograph
- Column: Xterra ® MS C-18, 50 mm long, 4.6 mm i.d., 3.5 µm particle size
- Cooler Temperature: 15°C
- Mobile Phase: Pump A: 0.1 % Formic Acid in Milli-Q water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow rate: 0.5 mL/min
- Injection volume: 10 µL

MS Conditions
- Scan type: MRM
- Ionization mode: ESI +ve (polarity)

- Q1/Q3: 230.00/174.05
  230.00/96.15
  230.00/68.00

Source parameters

- Nebulizing gas flow: 3.00 L/min
- Heating gas flow: 10 L/min
- Interface Temperature: 350°C
- DL Temperature: 250°C
- Heat block Temperature: 400°C
- Drying gas flow: 10.00 L/min
- Collision energy (CE): -15.0
  -26.0
  -38.0
- Q1 Pre Bias (v): -20.0
  -20.0
  -20.0
- Q3 Pre Bias (v): -12.0
  -20.0
  -26.0

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections, using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.
The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity
Diluent and matrix (after processing as per the method being validated) were injected into LC-MS/MS and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.

Detector response was linear in the range of 0.012 to 0.037 µg/mL with a regression coefficient (r) of 0.993. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was 0.304%.

Linearity

For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00250 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a stock solution of Terbuthylazine standard concentration 100 µg/mL.

Further, an aliquot of 0.5 mL of the standard stock solution was transferred to separate 50 mL volumetric flask, then the volume was made up to mark with diluent and mixed well. This resulted in the DLC Stock solution of concentrations of 1.000 µg/mL.

For detector linearity, a series of five working standard solutions in the range of 0.0120 to 0.0380 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of DLC stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)
DLC1	1.000	0.120	10	0.0120
DLC2		0.180	10	0.0180
DLC3		0.250	10	0.0250
DLC4		0.310	10	0.0310
DLC5		0.380	10	0.0380

The linearity solutions, prepared as above, were injected to LC-MS/MS in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

In addition, the regression residual (di) was measured as below:

$$di = Yi - Yyi$$

where:
Yi – measured value
Yyi – estimated value which corresponds to yi and I derived from the calibration function.
The regression residuals were represented in the residual plot.

Accuracy and Precision

Test item concentrations in the test samples at two levels (low and high dose) were analysed in six replications (two replication each from top, middle and bottom layers) using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/L)}{Nominal\ test\ item\ concentration\ (mg/L)} \times 100$$

$$Nominal\ Concentration(mg/L) = \frac{Weight\ of\ test\ item(g)taken\ for\ test\ sample\ preparation}{Volume\ of\ Matrix\ (mL)} \times 10^6$$

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range, 70.0% to 120.0% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.

The accuracy of the method, as mean percent recovery at 0.051 mg ai/kg and 120.711 mg ai/kg fortification levels were 93.464 and 96.376 with a precision (as % RSD) of 6.852 and 2.181, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as %RSD to be < 20.0%.

Stability

Stability of test item in the matrix was performed at low dose and high dose level.
The test samples after sampling for accuracy/precision test were retained at room temperature till the completion of stability test.
Samples were taken for analysis at the following intervals:

- 0 hour (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
- At 4 hours (samples stored at room temperature)

On each stability interval three replications (one replication each from top, middle and bottom layers) for each dose group (low and high) was taken and analysed for test item concentration.
Stability of the test sample was considered acceptable as the recoveries are within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

Analytical Solution (Injection Medium) Stability

Final extract stability: The solution stability of sample solutions
corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 145 hours and 169 hours.
The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 145 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions (working standard solution, and sample solutions corresponding to dose formulation concentrations) stored at ambient temperature for 145 and 169 hours.
The % difference for the standard solutions while stored at ambient temperature for 145 hours was -0.602%.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 145 and 169 hours.
The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.
The %difference in all cases were within the study plan acceptance criteria of ± 10.0% of initial value indicating that the sample solution was stable up to 169 hours when stored at room temperature.

Stability Test

The stability of the test item in the test sample was evaluated for nominal dose concentration at 0.051 mg ai/kg and 120.250 mg ai/kg fortification levels in the matrix. The concentrations of the test items in the dose formulations were determined after 4 hours for the dose formulation stored at room temperature

condition.
The mean percent recovery in the test item concentrations in the dose formulation stored at room temperature after 4 hours were 96.078 and 93.014 with % RSD 2.041 and 2.527 at the dose concentration 0.051 mg ai/kg and 120.250 mg ai/kg, respectively.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.012 to 0.037 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e 0.012 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration used in the accuracy and precision was considered as LOQ. i.e. 0.051 mg ai/kg.

System Suitability and Standard Check
System suitability and standard check was performed on all the days of analysis and the obtained results met the acceptance criteria.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix: in 50% w/v Sucrose solution in Milli-Q water.

A 2.1.1.2.10 HPLC with UV/PDA detector (in deionized water)

A 2.1.1.2.10.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/10 (filed as KCP 10.6.2/01)
Report	H-01-2022: SEEDLING EMERGENCE AND SEEDLING GROWTH TEST WITH TERRESTRIAL PLANTS, Report No.: AG-G1156, VISHALA N., 2023
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The HPLC method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range and precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The solution stability of working standard and processed sample solutions under specified storage conditions was also assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the ma-

trix- deionized water.

- Low dose – 5.5955 mg/L (0.005 mL formulated product/L)
- High dose – 11191.000 mg/L (10 mL formulated product/L)

Note: After applying density (1.119 g/mL):

- 0.005 mL formulated product/L (Low dose) = 5.5955 mg formulated product/L.
- 10 mL formulated product/L (High dose) = 11190 mg formulated product /L.
- LOQ level for active ingredient is 2.5879 mg/L

Preparation of Test Sample

Accurately 1.1191 g of test item was weighed into a 100 mL volumetric flask. Then the volume was made up with the matrix and sonicated for 5 minutes. This results in a solution of concentration of 11191.00 mg/L and was labelled as 'High dose'.

An aliquot of 0.05 mL of 'High dose' was transferred into 100 mL volumetric flask and volume was made up with reconstituted water, This results in a solution of concentration of 5.5955 mg/L and was labelled as 'Low dose.'

Preparation of Test Sample Solutions

Samples from the test samples in five composite replications for method validation was taken diluted using diluent such a way to get the final concentration of approximately 1.3 µg/mL of Terbuthylazine.

Low Dose (5.5955 mg/L): Diluted 5 mL of the sample to 10 mL volumetric flask and diluted with diluent to obtain the resulting solution of concentration of approximately 1.3 µg/mL and injected to HPLC.

High Dose (11191.000 mg/L): Diluted 1 mL of the sample to 50 mL volumetric flask and diluted using diluent. Further 0.63 mL of this solution was transferred to 50 mL volumetric flask and made up to the mark using diluent to obtain the resulting solution of concentration of approximately 1.3 µg/mL and injected to HPLC.

Similarly, one control sample was processed (similar to low dose and high dose) and analysed.

Chromatographic Conditions

- Instrument: High performance liquid chromatography equipped with UV/PDA detector
- Column: Shimpack C 18, 250mm long, 4.6 mm i.d, 5µm particle size
- Cooler Temperature: 15°C
- Column Temperature: 25°C
- Mobile Phase: Pump A: 0.1% Orthophosphoric acid in Milli-Q Water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow Rate: 1.0 mL/min
- Injection Volume: 10 µL
- Run time: 10 minutes

All the parameters were maintained constant throughout the analysis.

The chromatographic system was calibrated before and after a set of sample injections using external working standard solution.

Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).

The peak area of analyte for each injection was recorded.

The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity

Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into HPLC and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.

Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at

the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.0660 to 1.9595 µg/mL with a regression coefficient (r) of 0.9996. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low dose (at injection) and this was used as diluent, to prepare the matrix matched standard.
Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was 4.48%

Linearity
For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00254 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a DLC stock solution of Terbuthylazine standard concentration 99.974 µg/mL.
For detector linearity, a series of five working standard solutions in the range of 0.0660 to 1.9595 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of DLC stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)	Range corresponding to original sample (mg Terbuthylazine/L)	Range corresponding to original sample (mg Product/L)
DLC1	99.974	0.033	50	0.0660	0.132	0.285
DLC2		0.340	50	0.6798	1.360	2.940
DLC3		0.650	50	1.2997	2.599	5.620
DLC4		0.820	50	1.6396	3.279	7.090
DLC5		0.980	50	1.9595	3.919	8.474

The linearity solutions, prepared as above, were injected to HPLC in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision
Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range, 70% to 120% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.
The accuracy of the method, as mean percent recovery at 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L fortification levels were 97.302 and 86.577 with a precision (as % RSD) of 2.694 and 1.714, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as % RSD to be < 20.0%.

Analytical Solution (Injection Medium) Stability
Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24hours and 121 hours. The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions working standard solution stored at ambient temperature after 24 hours and sample solutions corresponding to dose formulation concentrations stored at ambient temperature after 24 hours and 121 hours.
The % difference for the standard solutions while stored at ambient temperature for 24 hours was -0.64%.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours and 121 hours.
The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.
The %difference in all cases were within the study plan acceptance criteria of ± 10.0% of initial value indicating that the standard solution was stable up to 24 hours when stored at room temperature.

Stability Test
The test samples after sampling for accuracy/precision test were retained at room temperature till the completion of stability test.
Samples were taken for analysis at the following intervals:

- 0 hour (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
- At 24 hours (samples stored at room temperature)

On 24hour stability interval three composite samples (except on 0-hour analysis) for each dose group (low and high) was drawn/taken and analysed for test item concentration.
Stability of the test sample was considered acceptable as the recoveries are within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

The stability of the test item in the test sample was evaluated for nominal dose concentration at 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L fortification levels in the matrix. The concentrations of the test items in the dose formulations were determined after 24 hours for the dose formulation stored at room temperature condition.
The mean percent recovery in the test item concentrations in the dose formulation stored at room tem-

perature after 24 hours were 103.242 and 91.509 with % RSD 1.149 and 0.603 at the dose concentration of 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L, respectively.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0660 to 1.9595 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e 0.660 µg/mL was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria. The lowest dose concentration without applying analyte content used in the accuracy and precision test was 5.5955 mg/L.
The LOQ was calculated by considering the active content in the test item i.e. 2.5879 mg Terbuthylazine/L.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of analytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix: deionized water.

A 2.1.1.2.11 HPLC with PDA detector (in deionized water)

A 2.1.1.2.11.1 Method validation

Comments of Evaluator:	Method is accepted

Reference:	KCP 5.1.2/11 (filed as KCP 10.6.2/02)
Report	H-01-2022: VEGETATIVE VIGOUR TEST, Report No.: AG-G1157, VISHALA N., 2023
Guideline(s):	SANTE/2020/12830, rev.2
Deviations:	No
GLP:	Yes
Acceptability:	Yes

Materials and methods

The HPLC method for the analysis of test item in test samples was validated by assessment of the specificity, linearity, range and precision, accuracy, LOD (Limit of Detection), LOQ (Limit of Quantification) and matrix effect. The solution stability of working standard and processed sample solutions under specified storage conditions was also assessed.

Fortification levels for test samples
Method validation and stability test was carried out at the following test item concentrations in the matrix - deionized water.

- Low dose - 2.5879 mg Terbuthylazine /L
- High dose - 5175.8375 mg Terbuthylazine /L

Preparation of working standard

Working standard solution of concentration, approximately 1.3 µg/mL of Terbuthylazine was prepared.
Standard stock solution: Approximately 0.0025g of reference standard was weighed into a 25 mL volumetric flask. Then volume was made up to the mark using diluent and mixed thoroughly. This results in a stock solution of approximately 100 µg/mL.
Working Standard Solution: Aliquots of 0.65 mL of the stock solution was transferred to 50 mL volumetric flasks and diluted using diluent. This resulted in the working standard solution of approximately 1.3 µg/mL of Terbuthylazine.
Prepared two working standard solutions (starting from different weighing) and used one for system suitability test and the other for sample analysis (as bracketing calibration standards).

Chromatographic Conditions

- Instrument: High performance liquid chromatography equipped with PDA detector
- Column: Inertsil ODS 3V, 250mm long, 4.6mm i.d, 5µm particle size
- Cooler Temperature: 15°C
- Column Temperature: 25°C
- Wavelength: 210 nm
- Mobile Phase: Pump A: 0.1% Orthophosphoric acid in Milli-Q Water
  Pump B: Acetonitrile
- Mobile Phase Ratio: Pump A : Pump B (30:70% v/v)
- Flow Rate: 1.0 mL/min
- Injection Volume: 10 µL

All the parameters were maintained constant throughout the analysis.
The chromatographic system was calibrated before and after a set of sample injections using external working standard solution.
Analyte peak was identified in the sample by comparing its retention time with that of analyte peak in the reference standard (check for the absence of such a peak in control).
The peak area of analyte for each injection was recorded.
The peak areas of two working standard solutions (injected before and after a set of sample injections) was averaged and used for calculating the concentration of test item in the samples.

Validation

Specificity
Diluent (Acetonitrile) and matrix (after processing as per the method being validated) were injected into HPLC and interference of peak at the retention time of analyte was absent. This ensures the specificity of the method.
Specificity of the method was acceptable as the diluent blank and matrix blank showed no interference at the retention time of the analyte in the analysis and the obtained results met the study plan acceptance criteria of absence of interference at the retention time of the analyte.
Detector response was linear in the range of 0.0660 to 1.9595 µg/mL with a regression coefficient (r) of 0.9996. This result met the acceptance criteria of a linear regression coefficient (r).

Matrix effect
Assessment of matrix effects was performed by comparing the analyte response of one individual standard prepared in blank matrix was compared with one prepared in solvent. The matrix effect was calculated as below.

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

Matrix effects are considered significant if they exceed ± 20%. Matrix matched calibration should be used if significant matrix effects occur.
The matrix was diluted suitably with diluent to the dilutions level, similar to the prepared solutions of low

dose (at injection) and this was used as diluent, to prepare the matrix matched standard.
Matrix effects was performed by comparing the analyte response one individual standard prepared in blank matrix was compared with one prepared in solvent. The obtained matrix effect was 4.48%

Linearity
For detector linearity, standard stock solutions were prepared by transferring an accurate quantity of 0.00254 g of Terbuthylazine (purity: 98.4 %) into a 25 mL volumetric flask, volume was made up to the mark with diluent and mixed thoroughly. This resulted in a DLC stock solution of Terbuthylazine standard concentration 99.974 µg/mL.
For detector linearity, a series of five working standard solutions in the range of 0.0660 to 1.9595 µg/mL standard were prepared by diluting a known volume of DLC Stock solution with diluent as given below.

Test	Conc. of DLC Stock solution taken (µg/mL)	Volume of DLC stock solution taken (mL)	Volume made up to (mL)	Conc. of solution (µg/mL)	Range corresponding to original sample (mg Terbuthylazine/L)	Range corresponding to original sample (mg Product/L)
DLC1	99.974	0.033	50	0.0660	0.132	0.285
DLC2		0.340	50	0.6798	1.360	2.940
DLC3		0.650	50	1.2997	2.599	5.620
DLC4		0.820	50	1.6396	3.279	7.090
DLC5		0.980	50	1.9595	3.919	8.474

The linearity solutions, prepared as above, were injected to HPLC in duplicate. A graph of detector response versus concentration was plotted and regression coefficient (r) was established, for the selected range. The method was considered acceptable as the r value was not less than 0.99.

Accuracy and Precision
Test item concentrations in the test samples at two levels (low and high dose) were analysed in five composite replications samples using the method being validated.
Accuracy as percent recovery was calculated for analyte as per following formula:

$$\%\ Recovery = \frac{Analysed\ test\ item\ concentration\ (mg/Kg)}{Nominal\ test\ item\ concentration\ (mg/Kg)} \times 100$$

$$Nominal\ Concentration(mg/Kg) = \frac{Weight\ of\ test\ item(mg)taken\ for\ test\ sample\ preparation}{Weight\ of\ Matrix(Kg)}$$

The method was considered acceptable, as the mean percent recovery was in the range 70% to 120% (SANTE/2020/12830 Rev.2) at each concentration levels for each analyte.

The precision as % RSD was calculated as per following formula:

$$\%RSD = \frac{SD}{Mean} \times 100$$

Precision of the method was acceptable as %RSD from five replicates at each fortification level was less than 20.0%.
The accuracy of the method, as mean percent recovery at 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L fortification levels were 97.302 and 86.577 with a precision (as % RSD) of 2.694 and 1.714, respectively. The obtained results met the acceptance criteria of recoveries to be within 70% to 120% and precision as % RSD to be < 20.0%.

Analytical Solution (Injection Medium) Stability

Final extract stability: The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24hours and 121 hours. The processed sample solutions were considered stable as the obtained recoveries are within 70-120%.
Standard stability: The solution stability of the existing standard was measured against freshly prepared standards by comparing the detector response. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours.
The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%.

The solution stability was evaluated for the analytical solutions working standard solution stored at ambient temperature after 24 hours and sample solutions corresponding to dose formulation concentrations stored at ambient temperature after 24 hours and 121 hours.
The % difference for the standard solutions while stored at ambient temperature for 24 hours was -0.64%.
The solution stability of sample solutions corresponding to low dose and high dose was measured by re-injection (triplicates) of the processed solution against freshly prepared standards. The stability was evaluated for the solutions stored at ambient temperature after at 24 hours and 121 hours.
The processed sample solutions were considered stable, as the obtained recoveries were within 70-120%.
The %difference in all cases were within the study plan acceptance criteria of ± 10.0% of initial value indicating that the standard solution was stable up to 24 hours when stored at room temperature.

Stability Test
The test samples after sampling for accuracy/precision test were retained at room temperature till the completion of stability test.
Samples were taken for analysis at the following intervals:

- 0 hour (overall mean concentration obtained for Accuracy/Precision test was used as concentration at '0' hour)
- At 24 hours (samples stored at room temperature)

On 24hour stability interval three composite samples (except on 0-hour analysis) for each dose group (low and high) was drawn/taken and analysed for test item concentration.
Stability of the test sample was considered acceptable as the recoveries are within the acceptable range of 70-120%, measured against freshly prepared standards and %RSD was less than 20.0%.

The stability of the test item in the test sample was evaluated for nominal dose concentration at 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L fortification levels in the matrix. The concentrations of the test items in the dose formulations were determined after 24 hours for the dose formulation stored at room temperature condition.
The mean percent recovery in the test item concentrations in the dose formulation stored at room temperature after 24 hours were 103.242 and 91.509 with % RSD 1.149 and 0.603 at the dose concentration of 2.5879 mg Terbuthylazine/L and 5175.8375 mg Terbuthylazine/L, respectively.

Limit of detection and limit of quantification
The lowest calibration standard used in Linearity test is considered as Limit of detection (LOD). A calibration curve was done using 5 calibration solutions of range 0.0660 to 1.9595 µg/mL used in the method. Each solution was injected to the analytical instrument in duplicate The lowest calibration standard used in the linearity and range test i.e 0.132 mg/L was considered as the LOD.

The lowest dose concentration (in terms of active substance) used in the accuracy and precision tests was considered as LOQ provided that the specificity, accuracy and precision test results at this level meet the specified acceptance criteria.
The LOQ was calculated by considering the active content in the test item i.e. 2.5879 mg Terbuthylazine/L.

Conclusion

The method was fully validated according to SANTE/2020/12830, rev.2. Results of the validation of ana-

lytical method was confirmed that this method is suitable for analysis the content of the active substance terbuthylazine in matrix: deionized water.

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

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

No new or additional studies have been submitted.

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

Not relevant.