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Battelle UK

FINAL REPORT

Method Validation – Analytical Method for the Determination of Residues of Strychnine in Soil

DATA REQUIREMENT AND TEST GUIDELINES

OECD Guidance Document on Pesticide Residue Analytical Methods ENV/JM/MONO(2007)17

EC Guidance Document SANCO/825/00 rev.8.1 (16/11/2010)

EPA Ecological Effects Test Guidelines OCSPP 850.6100

ABBREVIATIONS AND SYMBOLS

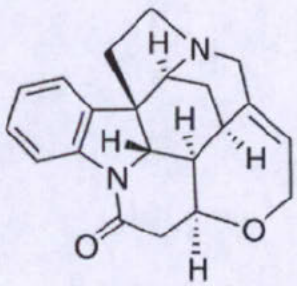
Abbreviation	Definition
CAS	Chemical Abstracts Service
CoA	certificate of analysis
conc.	concentration
Dil	dilution
EC	European Commission
ESP+	positive ion electrospray (mass spectrometry)
EU	European Union
GLP	Good Laboratory Practice(s)
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
HPLC	high performance liquid chromatography
LC-MS/MS	tandem liquid chromatograph-mass spectrometer-mass spectrometer
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MS	mass spectrometry
n	number of data
NA	not applicable
ND	not detected
O.D.E	Oven dried equivalent
OECD	Organisation for Economic Cooperation and Development
QAU	quality assurance unit
R (or r)	correlation coefficient
RSD	relative standard deviation
SD	standard deviation
STD	standard
UPW	ultra-pure water
V_f	final volume of the sample in mL
v/v	volume for volume
w/w	weight for weight

2. OBJECTIVE

The purpose of this study was to develop and validate a method in terms of linearity, specificity, accuracy and precision for the determination of residues of strychnine according to the OECD Guidance Document ENV/JM/MONO(2007)17 [1], EC Guidance Document SANCO/825/00 rev. 8.1 [2] and EPA Test Guidelines OCSPP 850.6100 [3].

3. TEST ITEM

3.1. Strychnine

Common Name:	Strychnine
CAS No.:	57-24-9
Molecular Formula:	$C_{21}H_{22}N_2O_2$
Molecular Weight:	334.41 g/mol
Molecular Structure:	
Supplier:	Sigma-Aldrich
Batch No.:	SZBE079XV
Purity:	99.9%
Expiry Date:	20 March 2019
Storage Conditions:	Ambient

4. TEST SYSTEM

Untreated sandy clay loam soil was sourced commercially from Agvise. The certificate of analysis of the soil sample is presented in Appendix 2.

The moisture content of the soil was determined to be 16% at Battelle UK prior to use. All calculations are based on the oven dried equivalent (O.D.E) weight of the soil.

Except for the removal of a sub-sample for analysis, all specimens were stored in a refrigerator at nominally 4°C.

5. METHOD VALIDATION

The method was validated in terms of linearity, specificity, accuracy and precision. The LOQ was established and the LOD was set as the lowest calibration standard of the method. Matrix effects and stability of the analyte in solvent and extract were also investigated.

Samples were fortified as described in the following table:

Matrix Sample	Untreated Control	Replicates at LOQ Fortification Level	Replicates at LOQ × 100 Fortification Level	Reagent Blank
Soil	2	6 at 0.05 mg/kg	6 at 5.00 mg/kg	1

5.1. Linearity

The linearity was investigated with solvent solutions of strychnine that were prepared in the range of 0.15 to 12.0 ng/mL (equivalent to 0.0075 to 0.60 mg/kg in samples, or 0.075 to 6.0 mg/kg when taking a dilution factor of 10 into account).

5.2. Accuracy

The accuracy was determined from the analysis of six replicates of fortified control samples at the LOQ and at 100 x LOQ.

5.3. Precision

The precision of the method was determined by measuring the relative standard deviation at each fortification level from six replicates of untreated soil samples spiked with strychnine at the LOQ and at 100 x LOQ.

5.4. Specificity

Specificity was confirmed by the use of LC-MS/MS which is a highly specific technique. One reagent blank and duplicates of untreated soil samples were analysed to demonstrate that no interferences greater than 30% of the LOQ were present at the retention time of the analyte.

5.5. Confirmation

Two structurally significant ion mass transitions were monitored, one for quantification purposes and one for confirmation purposes. Accuracy and precision data for both transitions were reported.

5.6. Matrix Effects

Matrix effects were assessed at the LOQ and at 100 x LOQ by comparing the response between solvent solutions and matrix-matched calibration solutions prepared at the same concentrations.

5.7. Stability

One sample extract, originally prepared during method validation and stored at a nominal temperature of 4°C for 3 days, was re-diluted with 10 mM ammonium formate (aq, pH4) and quantified against a set of freshly prepared calibration solutions to assess analyte stability in the presence of matrix.

Two stock solutions, prepared in methanol and stored at a nominal temperature of 4°C for 15 and 74 days, were analysed and compared against a freshly prepared stock solution to assess analyte stability in solvent. One fortification solution, prepared in methanol, was also analysed and compared against a fresh standard solution after storage of 3 days. All solutions were diluted to 1.0 ng/mL with 10 mM ammonium formate (aq, pH4) and injected in duplicate.

5.8. Limit of Detection

The limit of detection was set as the lowest calibration standard of the method.

6. ANALYTICAL METHODOLOGY

6.1. Equipment and Reagents

See Appendix 3 for full details of all equipment and reagents used in this study.

6.2. Sample Preparation and Processing

No preparation of the soil was required prior to its use within this study.

6.3. Standards, Fortifications and Calibrations

Stock solutions

Two stock solutions, each containing 1000 µg/mL of strychnine, were prepared by dissolving approximately 10 mg of the test item in appropriate amounts of methanol.

One stock solution (A) was used for fortification purposes. The second (B) was used to prepare calibration solutions.

A third stock solution, containing 1000 µg/mL of strychnine, was prepared in a similar manner for stability testing purposes.

Fortification

Fortification solutions, containing strychnine at 1.0 and 100 µg/mL, were prepared from the stock solution (A) by appropriate dilutions with methanol.

Six replicates of untreated soil samples were fortified with strychnine at the LOQ of 0.05 mg/kg by spiking an aliquot of 2 g (O.D.E) of soil with 0.10 mL of the fortification solution at 1.0 µg/mL.

Six replicates of untreated soil samples were fortified with strychnine at 100 x LOQ of 5.00 mg/kg by spiking an aliquot of 5 g (O.D.E) of soil with 0.10 mL of the fortification solution at 100 µg/mL.

Calibration

An intermediate solution, containing strychnine at 10 µg/mL, was prepared from the stock solution (B) by appropriate dilutions with methanol. Two working solutions, containing strychnine at 5.0 and 50 ng/mL, were prepared by successive dilutions of the 10 µg/mL solution with 10mM ammonium formate (aq, adjusted to pH 4 with formic acid).

Solvent calibration solutions containing strychnine were prepared by mixing and diluting appropriate amounts of the working solutions with 10mM ammonium formate (aq, adjusted to pH 4 with formic acid). The concentrations ranged from 0.15 to 12.0 ng/mL of strychnine.

Two matrix-matched standards, containing strychnine at 1.0 and 10 ng/mL, were prepared in a similar way to the solvent equivalent standards with the addition of 0.10 mL of control matrix before completing to 1.0 mL with 10mM ammonium formate (aq, adjusted to pH 4 with formic acid). These were used to assess matrix effects.

Details of preparation of solvent and matrix standards used in this study is given in Table 1.

All solutions were stored at nominally 4°C when not in use.

6.4. Analytical Procedure

A 2 g (O.D.E) quantity of soil sample was weighed into a 50 mL centrifuge tube. Recovery efficiency samples were fortified as necessary at this stage. A volume of 10 mL of 2M sodium hydroxide (aq) solution was added and the sample was shaken on a wrist action shaker for 20 minutes. An aliquot of 10 mL of 5% ethanol in ethyl acetate was added to the sample followed by 10 mL of hexane. The sample was shaken for another 20 minutes, followed by centrifugation at 3000 rpm for three minutes. The supernatant was transferred to a 40 mL glass vial and the soil was extracted once more with 10 mL of 2M sodium hydroxide (aq), 10 mL of 5% ethanol in ethyl acetate and 10 mL of hexane. After centrifugation, the supernatants were combined in the glass vial and evaporated to dryness under a stream of nitrogen at 40°C. The dried residues were reconstituted with 5 mL of 0.1% formic acid in methanol by sonication, followed by the addition of 5 mL of 10 mM ammonium formate (aq, pH4). The sample was mixed again by sonication and vortex.

For 100xLOQ samples, an aliquot of 0.10 mL was diluted with 0.90 mL of control matrix. An aliquot of 0.10 mL of each control, LOQ and diluted 100xLOQ sample was transferred into an autosampler vial and diluted with 0.90 mL of 10 mM ammonium formate (aq, pH4).

All samples were analysed by LC-MS/MS.

A reagent blank was also extracted and analysed in the same way.

The analytical method flow chart is presented in Figure 1.

6.4.1. Time Required for Analysis

The methodology is normally performed with a batch of 15 samples. One person can complete the extraction of 15 samples in nine hours, including the preparation of solvents, reagents and calibration standards and weighing of samples.

However, the weighing and solvent preparation may be performed before the day of extraction in order to complete the analytical procedure in one day (7.5 hours).

6.4.2. Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

6.5. LC-MS/MS Analysis

All samples were analysed by liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS).

6.5.1. Instrument Description

Pump	Agilent 1290 series binary pump
Column Oven	Agilent 1290 series
Autosampler	Eksigent (Presearch) HTS-xt
Detector	API 6500 LC-MS/MS System with Q Jet Ion Guide
Gas Supply	Peak Scientific nitrogen generator and HPC air generator

6.5.2. Liquid Chromatography Conditions

Mobile Phase A	10 mM ammonium formate (aq), adjusted to pH4 with formic acid		
Mobile Phase B	0.1% formic acid in methanol		
Gradient	Time [min]	%A	%B
	0	100	0
	1.0	100	0
	2.0	0	100
	4.0	0	100
	5.0	100	0
	6.0	100	0
Flow Rate	1.0 mL/min		
Injection Volume	10 µL		
Autosampler Wash Cycle[1]	Wash 1 = DMSO:IPA:MeCN:MeOH (1:1:1:1, v/v/v/v)	Valve Clean 60 s	Post Clean 15 s
		Stator Wash Time 30 s	
	Wash 2 = Water/MeOH (9:1 v/v)	Valve Clean 30 s	Post Clean 15 s
		Stator Wash Time 30 s	
Column	Agilent Zorbax Eclipse XDB-C18 5 µm, 150 x 4.6 mm		
Column Oven	30 ± 1°C		
Switching Valve	Time [min]	Position	Flow Direction
	0.0	B	To Waste
	2.0	A	To Mass Spec
	4.5	B	To Waste

Under these conditions the retention time of strychnine is approximately 3.3 ± 0.5 minutes.

[1] During method development, it was noted that the analyte was susceptible to carryover therefore the wash cycle has been developed and recommended to reduce this. As this may be instrument specific, each laboratory performing this method should assess the carryover effect on their system and adjust the wash cycle as required.

6.5.3. Mass Spectrometry Conditions

Ion Source	Positive Ion Turbo Spray Ionisation	
Curtain Gas [CUR]	20 (arbitrary units)	
Temperature [TEM]	500	
Ion Transfer Voltage [IS]	5500	
Collision Gas Cell [CAD]	-2.00 (arbitrary units)	
GS1 Nebuliser Gas	60 (arbitrary units)	
GS2 Turbo Gas	50 (arbitrary units)	
Interface Heater [ihe]	On	
CEM [Electron Multiplier]	1600	
Scan Type	MRM	
MRM Conditions	Strychnine Transition 1	Strychnine Transition 2
Q1 m/z	335	335
Q3 m/z	156	184
Dwell Time	150 ms	150 ms
Declustering Potential [DP]	110	110
Entrance Potential [EP]	10	10
Collision Energy [CE]	63	53
Collision Cell Exit Potential [CXP]	10	12

7. CALIBRATION AND CALCULATION

Calibration solutions were prepared for strychnine at concentrations ranging from 0.15 to 12.0 ng/mL in solvent (equivalent to 0.0075 to 0.60 mg/kg in samples, or 0.075 to 6.0 mg/kg when taking dilution factor of 10 into account for 100xLOQ samples).

Multi-level calibration curves of the form $y = mx + c$ were obtained using solvent calibration solutions. The calibration curves were constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression, with a weighting factor of 1/x. Correlation coefficients, r, greater than 0.995 were achieved for both transitions over the concentration range tested. The quantification of analyte in the final sample extract was made by comparison to the calibration curve.

The concentration of strychnine in a sample extract was calculated as follows:

$$\text{Strychnine concentration} = \left(\frac{[P - c] \times D \times V \times F}{m \times W \times A \times 1000} \right) \quad [\text{mg/kg}]$$

Where:

P	=	analyte peak area
m	=	slope of the calibration curve
c	=	intercept of the calibration curve
D	=	dilution factor (1 or 10)
V	=	extraction volume (10 mL)
F	=	final volume (1 mL)
W	=	sample weight (2 g, O.D.E)
A	=	aliquot volume (0.10 mL)

The recovery efficiencies in the fortified samples were calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found [mg/kg]}}{\text{Amount spiked [mg/kg]}} \times 100\%$$

TABLES

The tables in this report have been computer generated. The results presented below are rounded values of those held in memory but the results are derived from calculations that were performed without rounding. Consequently, there may be minor apparent differences between the reported results and calculations using rounded values. Any such difference is not considered significant.

Table 1: Typical Preparation of Calibration Standard Solutions in Solvent and Matrix

Calibration Standard Concentration (ng/mL)	Working Solution Used (ng/mL)	Aliquot of Working Solution (mL)	Aliquot of Mobile Phase A* (mL)	Final Volume (mL)
0.15	5.0	0.03	0.97	1.0
0.30	5.0	0.06	0.94	1.0
1.0	5.0	0.20	0.80	1.0
2.5	50	0.05	0.95	1.0
5.0	50	0.10	0.90	1.0
7.5	50	0.15	0.85	1.0
10	50	0.20	0.80	1.0
12	50	0.24	0.76	1.0

Calibration Standard Concentration (ng/mL)	Working Solution Used (ng/mL)	Aliquot of Working Solution (mL)	Aliquot of Control Extract (mL)	Aliquot of Mobile Phase A* (mL)	Final Volume (mL)
1.0	5.0	0.20	0.10	0.70	1.0
10	50	0.20	0.10	0.70	1.0

* = 10 mM ammonium formate (aq), adjusted to pH 4 with the addition of formic acid

Pro-rata volumes and concentrations may have been used. The table shows the typical solutions prepared and is not an exhaustive list of all standards that were prepared within the study.

FIGURES

Figure 1: Analytical Method Flow Chart

EXTRACTION

- Weigh 2.0 g (O.D.E) of homogenised soil into a 50 mL centrifuge tube
- Fortify if necessary and allow the solvent to soak into the matrix
- Add 10 mL of 2M NaOH (aq) followed and shake for 20 minutes using a wrist action shaker
- Add 10 mL of 5% ethanol in ethyl acetate and 10 mL of hexane
- Shake for another 20 minutes on a wrist action shaker
- Centrifuge at 3000 rpm for 3 minutes and collect the top layer into a 40 mL glass vial
- Repeat the extraction by adding another 10 mL of 2M NaOH (aq) and shake for 20 minutes
- Add 10 mL of 5% ethanol in ethyl acetate and 10 mL of hexane and hake for another 20 minutes on a wrist action shaker
- Centrifuge at 3000 rpm for 3 minutes and combine the top layers in the glass vial
- Evaporate the sample to dryness under a stream of nitrogen at 40°C
- Reconstitute the dried residues with 5 mL of 0.1% formic acid in methanol
- Sonicate the sample for 5 minutes
- Add 5 mL mobile phase A*
- Sonicate the sample for 5 minutes and vortex mix for 1 minute

DILUTION

- For 100xLOQ samples, dilute by adding 0.1 mL of extract to 0.9 mL of control extract in an autosampler vial.
- For all samples, dilute 0.10 mL of extract with 0.90 mL of mobile phase A* in an autosampler vial
- Analyse by LC-MS/MS
- Solvent standards used

* = 10 mM ammonium formate (aq), adjusted to pH 4 with the addition of formic acid

Appendix 3: Details of Suppliers and Manufacturers of Equipment and Reagents (continued)

Reagents

Chemical	Supplier
Ammonium formate 99% Ethanol absolute analytical reagent grade Methanol HPLC grade Formic acid 99-100%	Fisher Scientific (Acros Organics, Fisons) Fisher Scientific UK Ltd Bishop Meadow Road Loughborough Leicestershire LE11 5RG
Hexane Chromosolv HPLC Grade ≥95% Ethyl Acetate Chromosolv HPLC Grade ≥99.7% Sodium hydroxide reagent grade ≥98% (pellets, anhydrous)	Sigma-Aldrich The Old Brickyard New Road Gillingham Dorset SP8 4XT
HPLC grade water	Rathburn Chemicals Ltd. Walkerburn, Scotland

Preparation of reagents

10 mM ammonium formate (aq), adjusted to pH 4 with formic acid – 0.63 g of ammonium formate was weighed into 1 L glass bottle and dissolved in 1 L of HPLC water. Formic acid 99-100% was added (approximately 0.4 mL required) to adjust to pH4, measured by a calibrated pH meter. Use within one week.

0.1% formic acid in methanol – 1 mL of 99-100% formic acid was added to 1L of methanol. Use within one month.

2M NaOH (aq) – 80 g of sodium hydroxide was weighed into a 1 L glass bottle and dissolved in 1 L of HPLC water. Use within one month.

5% ethanol in ethyl acetate – 50 mL of ethanol was added to 950 mL of ethyl acetate. Use within one month.

All solvents were stored ambient.