1. Introduction

The objective of this study was to validate methodology for the determination of residues of terbufos, terbufos sulfoxide and terbufos sulfone, in soil and sediment, and to demonstrate a suitable confirmatory technique.

To determine the validity of the analytical method, it was necessary to determine:

- recovery (accuracy)
- precision
- linearity
- specificity
- limit of detection
- limit of quantitation

This study was based upon fulfilling the requirements of the following regulatory guidelines:

Guidance for Generating and Reporting Methods of Analysis in Support of Residue Data Requirements for Annex II (part A, Section 4), and Annex III (part A, Section 5) of Directive 91/414, SANCO/3029/99 rev. 4.

SANCO/825/00 rev.8.1 of 16 November 2010 : Guidance document on residue analytical methods; to fulfil the post-registration monitoring data requirements for Annex II (part A, Section 4), and Annex III (part A, Section 5) of Council Directive 91/414/EEC as amended by Commission Directive 96/46/EC.

U.S. Environmental Protection Agency Residue Chemistry Test Guidelines OPPTS 860.1340 Residue Analytical Method.

Study organisation

The sample analysis took place at:

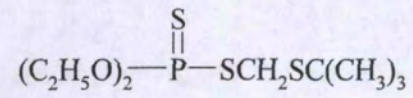
Huntingdon Life Sciences, Ltd.
Eye Research Centre
Eye
Suffolk
IP23 7PX
England

Relevant study dates

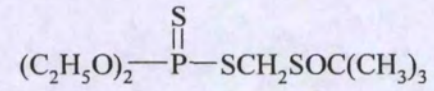
Study initiation	:	27 February 2012
Analytical phase commenced	:	1 March 2012
Analytical phase completed	:	13 March 2012

2. Materials

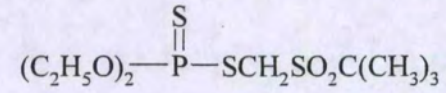
2.1 Analytical standard – terbufos

Identity	Terbufos
Chemical name (IUPAC)	S-tert-butylthiomethyl O,O-diethyl phosphorodithioate
Structure	
Storage conditions	Ambient
Batch number	0351H01HC
CAS number	13071-79-9
Purity	89.3%
Supplier	Sponsor
Re-test date	17 December 2012

2.2 Analytical standard – terbufos sulfoxide

Identity	Terbufos sulfoxide
Chemical name (IUPAC)	S-[(tert-butylsulfinyl)methyl] O,O-diethyl phosphorodithioate
Structure	
Storage conditions	Refrigerator (approx 4°C)
Batch number	AC11957-97B
CAS number	10548-10-4
Purity	95.8%
Supplier	Sponsor
Expiry date	1 January 2013

2.3 Analytical standard – terbufos sulfone

Identity	Terbufos sulfone
Chemical name (IUPAC)	S-[(tert-butylsulfonyl)methyl] O,O-diethyl phosphorodithioate
Structure	
Storage conditions	Refrigerator (approx 4°C)
Batch number	L67-206
CAS number	56070-16-7
Purity	99.5%
Supplier	Sponsor
Re-test date	1 April 2012

Certificates of Analysis are presented in Appendix 1.

2.4 Control matrices

The control matrices were obtained from previous studies where excess untreated control material was available (the soil samples were previously sieved to 2 mm). These samples were assigned unique identification numbers and stored at approximately 4°C prior to use as control samples in this study. The samples had been previously classified as below:

	Sample identification		
	11/00/11498 (220909S)	**11/00/11499 (E250501A)**	**11/00/4317 (080411A)**
% Clay	33	5.7	2
% Silt	17	8.7	2
% Sand	50	85.7	96
Organic carbon (% w/w)	4.0	0.5	0.7
pH (H_2O)	6.6	Not available	5.0
pH ($CaCl_2$)	6.3	4.3	4.4
Classification (USDA)	Sandy clay loam	-	-
Classification (UK)	Sandy clay	Sand	Sand (sediment)

3. Methods

3.1 Validation

Sub-samples (20 g) of each of the three matrix types were fortified with known concentrations of the analytes simultaneously and analysed according to the following regime:

2 sub-samples of untreated sample matrix
5 sub-samples of untreated sample matrix fortified at the LOQ (0.01 mg/kg)
5 sub-samples of untreated sample matrix fortified at 0.1 mg/kg

These samples were then analysed using the analytical methodology, with each sample injected onto the chromatographic system once.

3.2 Final extract stability

An experiment was set up to demonstrate the stability of the analytes under the typical storage conditions of the final extracts if they are not quantified immediately after preparation. Processed control extracts, fortified with the three analytes simultaneously, were stored at approximately -20°C in the dark (i.e. in a freezer).

Aliquots of each of the control sample extracts were fortified with the three analytes at a concentration of 2.5 ng analyte/mL of final extract. The concentration of analytes in the stored extracts was determined at day 0 and after 7 days. The concentration of the analytes in freshly fortified control extracts was also determined at the same time that the samples stored for 7 days were analysed.

3.3 Matrix effects

Any possible sample matrix effects were investigated by the comparison of the instrument response to the analytes in the fortified final extract samples with the response of the analytes in solvent based calibration standard solutions prepared at the same time.

3.4 Analytical method

Samples were extracted with methanol:water (90:10 v:v), and cleaned up using solid phase extraction (SPE) cartridges, prior to reconstitution in acetontrile:water (60:40 v:v). Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The ion transitions monitored for quantitation purposes were *m/z* 289>103, *m/z* 305>187 and *m/z* 321>171 for terbufos, terbufos sulfoxide and terbufos sulfone, respectively. To demonstrate possible confirmation of residues, alternative ion transitions, *m/z* 289>233, *m/z* 305>243 and *m/z* 321>265 were also monitored, respectively.

The analytical method used in the laboratory is presented in Appendix 3.

3.5 Fortification/calibration solutions

Individual stock standard solutions (1 mg/mL) of the three analytes were prepared by dissolving an accurately weighed amount in a suitable volume of acetonitrile, correcting for purity as appropriate. These stock solutions were further diluted with acetonitrile to produce mixed fortification solutions at 10 µg/mL, 1 µg/mL and 0.1 µg/mL concentrations.

The instrument calibration solutions, over the concentration range 0.1 ng/mL to 10 ng/mL, were prepared by serial dilution of the fortification solutions in acetonitrile:water (60:40 v:v), as detailed below:

Standard solution used (ng/mL)	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1000	0.1	10	10
1000	0.05	10	5
1000	0.025	10	2.5
10	1	10	1
10	0.75	10	0.75
10	0.5	10	0.5
10	0.25	10	0.25
1	1	10	0.1

The standard solutions used in this study were also used in other GLP studies being performed for the same Sponsor. The use of these standard solutions is fully traceable to the other studies and copies of the standard solution preparation are included in the raw data package for this study.

3.6 Calculation of results for validation samples

Test samples were quantified using the following equation:

$$\text{Residue found (mg/kg)} = x \times \frac{1}{M} \times D$$

Where x (residue concentration in final solution) was calculated using the linear regression

$$y = m\,x + c \qquad \text{where } x \text{ (concentration in ng/mL)} = \frac{y - c}{m}$$

c	=	intercept
m	=	slope
y	=	peak area of sample
M	=	matrix concentration (g/mL)
D	=	dilution factor

Example calculation of terbufos detected in sediment fortified at 0.1 mg/kg (analytical identification 11/00/4317 F0.1 A, analysis batch 1). The primary data for this sample is presented in Table 26, Appendix 2.

Linear regression $y = m\,x + c$

$$2.99235e5 = 78612.1x + 2423.9$$

where
$y = 2.99235e5$
$m = 78612.1$
$c = 2423.9$

$$\text{Therefore, concentration of terbufos } (x) = \frac{2.99235e5 - 2423.9}{78612.1} = 3.776 \text{ ng/mL}$$

Matrix concentration = 0.05 g matrix/mL final extract
Dilution factor = 1

$$\text{Terbufos detected (mg/kg)} = \frac{3.776 \text{ng/mL} \times 1}{0.05 \text{ g/mL}} = 75.5 \text{ ng/g} = 0.0755 \text{ mg/kg}$$

$$\text{Recovery (\%)} = \frac{0.0755 \text{ mg/kg} \times 100}{0.1 \text{mg/kg}} = 76\%$$

Appendix 3 Analytical Method

DETERMINATION OF TERBUFOS, TERBUFOS SULFOXIDE AND TERBUFOS SULFONE IN SOIL AND SEDIMENT

1. General principle

Samples are extracted using a methanol/water mixture. Following SPE cleanup, quantitation is performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

2. Apparatus, glassware etc

Balances (various ranges)
Volumetric flasks (various sizes)
Syringes (various sizes)
Volumetric pipettes (various sizes)
Polypropylene tubes (15 mL)
Polyethylene bottles (250 mL)
Measuring cylinders (various sizes)

3. Materials	**Typical Grade (or equivalent)**
Acetonitrile	HPLC
Ammonium formate	AR
Methanol	HPLC
Formic acid	AR
Water	HPLC
Oasis HLB cartridges (60 mg, 3 mL)	

4. Preparation of reagents

Preparation of methanol:water (90:10 v:v) - methanol (900 mL) is mixed thoroughly with water (100 mL).

Preparation of acetonitrile:water (60:40 v:v) - acetonitrile (600 mL) is mixed thoroughly with water (400 mL).

Preparation of acetonitrile:water (20:80 v:v) - acetonitrile (200 mL) is mixed thoroughly with water (800 mL).

Preparation of water:methanol:formic acid (90:10:0.1 v:v:v) containing 0.01M ammonium formate - methanol (100 ml), ammonium formate (0.6 g) and formic acid (1 ml) is added to HPLC water (900 ml) and mixed thoroughly prior to use.

Preparation of methanol:formic acid (100.0.1 v:v) - methanol (1000 mL) is mixed thoroughly with formic acid (1 mL).

Note: variable quantities of the above may be prepared by adjusting the constituent quantities accordingly.

5. Analytical standard solutions

An appropriate amount of the test substance (corrected for purity) is accurately weighed and dissolved in acetonitrile to give the individual stock standard solution (typically 1 mg/mL concentration). Appropriate dilutions of the stock standard solution are made with acetonitrile to give mixed fortification standard solutions (typically 10 µg/mL, 1 µg/mL and 0.1 µg/mL).

The mixed fortification solutions are progressively diluted with acetonitrile:water (60:40 v:v) to produce a series of instrument calibration solutions in the range 0.1 to 10 ng/mL.

6. Procedure

Note - In order to minimise any potential degradation of the analytes in the study samples for analysis, the extraction and clean-up procedures must be completed without any interruption.

6.1 Transfer a sub-sample of soil/sediment (20 g) to a 250 mL polyethylene bottle.
6.2 Add fortification solution at this stage if required.
6.3 Add methanol:water (90:10 v:v, 80 mL) and securely cap the bottle.
6.4 Place on a mechanical shaker and shake at approximately 200 rpm for approximately 30 minutes.
6.5 Centrifuge at approximately 3500 rpm for approximately 3 minutes, to separate the phases.
6.6 Decant the supernatant into a new 250 mL polyethylene bottle.
6.7 Repeat the extraction as steps 6.3 to 6.6, combining the extracts in the 250 mL bottle.
6.8 Dilute the extract to volume (200 mL) with methanol:water (90:10 v:v) and mix well.
6.9 Transfer an aliquot (2.5 mL) of the extract to a 15 mL polypropylene tube.
6.10 Add water (5 mL) and mix well.

SPE cleanup

6.11 Condition the Oasis HLB SPE cartridge with acetonitrile (3 mL) and water (3 mL), discarding the eluate.
6.12 Load the extract from step 6.10 onto the SPE cartridge, discarding the eluate.
6.13 Wash the cartridge with an aliquot (5 mL) of acetonitrile:water (20:80 v:v), discarding the eluate, allowing the cartridge to have air pumped through for approximately 30 seconds to remove excess solvent.
6.14 Elute the SPE cartridge with an aliquot (3 mL) of acetonitrile, collecting in a 15 mL polypropylene tube.
6.15 Dilute the final extract to volume (5 mL) with water. Final matrix concentration ≡ 0.05 g sample matrix / mL final extract.
6.16 Perform any further dilutions using acetonitrile:water (60:40 v:v), as required.
6.17 Quantify the samples by the use of LC-MS/MS.

7. Flow chart of analytical procedure

Transfer 20 g soil/sediment to a 250 mL bottle
↓
Fortify with analytical standard (if required)
↓
Add methanol:water (90:10 v:v), and extract by mechanical shake
↓
Transfer aliquot of combined extract to 15 mL tube and add water
↓
SPE cleanup with Oasis HLB cartridge
↓
Elution with acetonitrile (3 mL) and dilution to volume (5 mL) with water
↓
Quantitative analysis by LC-MS/MS

8. LC-MS/MS conditions

Instrument:	AB Sciex API 4000 (using Analyst 1.4.2 software)
Mode:	Ionspray positive
Ion monitoring details:	Terbufos: *m/z* 289>103 *m/z* 289>233 (confirmatory) Terbufos sulfoxide: *m/z* 305>187 *m/z* 305>243 (confirmatory) Terbufos sulfone: *m/z* 321>171 *m/z* 321>265 (confirmatory)
Column:	Acquity UPLC® BEH C_{18} (2.1 mm x 50 mm, 1.7 µm), or equivalent, column temperature 45°C
Mobile phase A:	Water:methanol (90:10 v:v) + 0.01M ammonium formate + 0.1% formic acid
Mobile phase B:	Methanol:formic acid (100:0.1 v:v)

Gradient:

Time	%A	%B
0	50	50
0.2	50	50
2.0	5	95
2.5	5	95
3	50	50
4	50	50

Cycle time:	4 min
Injection volume:	10 µL
Flow rate:	0.5 mL/min
Retention times:	Terbufos: approx. 1.8 minutes Terbufos sulfoxide: approx. 1 minute Terbufos sulfone: approx. 1 minute
LOQ:	0.01 mg/kg
LOD:	0.1 ng/mL (= 0.002 mg/kg in sample matrix)

NOTE – alternative instruments may also be used, operated under conditions that are considered to be equivalent to those described above. However, some differences may be observed in the resulting data, such as slight differences in analyte retention times, or the observed sensitivity of the ion transitions monitored.