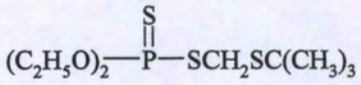
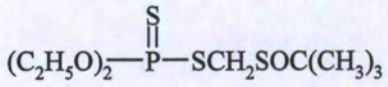
2. Materials

2.1 Analytical standards

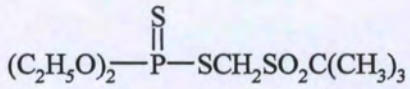
2.1.1 Terbufos

Identity	Terbufos (also known as Terbufos Technical)
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

2.1.2 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	01 January 2013

2.1.3 Terbufos Sulfone

Identity	Terbufos sulfone
Chemical name (IUPAC)	S-[(tert-butylsulfonyl)methyl] O,O-diethyl phosphorodithioate
Structure	
Storage conditions	Frozen (approx -20°C)
Batch number	L67-206
CAS number	56070-16-7
Purity	99.5%
Supplier	Sponsor
Re-test date	1 April 2017

The Certificates of Analysis are presented in Appendix 1.

2.2 Untreated samples

Untreated soil and sediment was obtained by the Department of Bioanalysis, Huntingdon Life Sciences for use in this study. Untreated soil samples were stored at ambient temperature, and sediment samples at approximately +4°C, prior to use.

The matrix samples were classified as follow:

	Bromsgrove soil 140708A	Evesham soil 050111A	Calwich Abbey Lake sediment 100412D
pH	6.2	7.9	7.6
Sand 2.00-0.05mm [USDA]	70	41	-
Sand 2.00-0.063[ADAS]	71	39	24
Silt 0.002 – 0.05mm [USDA]	17	28	-
Silt 0.063-0.002mm [ADAS]	16	31	63
Clay <0.002mm [ADAS]	13	30	13
Clay 0.002mm [USDA]	13	31	-
pH $CaCl_2$	5.6	7.3	7.0
Organic carbon by Wet oxidation	1.3	2.6	5.3
Textural class [USDA]	Sandy Loam	Clay loam	-
Textural class [ADAS]	Sandy Loam	Clay loam	Sandy Silt Loam

2.3 Reagents

A list of all reagents used is presented below:

Materials	Grade
Bromsgrove soil	Sandy loam soil
Evesham soil	Clay loam soil
Calwich Abbey Lake Sediment	Sediment (Sandy silt loam)
Acetonitrile	LC-MS grade
Ammonium formate	LC-MS grade
Methanol	LC-MS grade
Formic acid	LC-MS grade
Isopropanol	HPLC grade
Water	Ultra high purity (UHP)

2.4 Computer Systems

The computer system with version number used on this study are as follows:

Applied Biosystems/MDS Sciex Analyst (version 1.4.2 or later) to acquire and quantify data

Xybion Pristima (version 6.2) for Pharmacy test item management

3. Experimental procedures

3.1 Modifications to the supplied method

After an unsuccessful validation attempt in soil where low recoveries were obtained for the terbufos analyte (terbufos sulfoxide and terbufos sulfone recoveries were good in every batch analysed), the original validation laboratory was contacted with the Sponsor's permission to discuss possible reasons for this.

Following the discussions, a set of experiments were agreed to investigate the issue (these were; a) effect of increasing volume of elution solvent used, b) using an alternative dilution solvent and c) use of alternative elution solvents). Acceptable improvement in terbufos recovery was observed with option c), using an elution solvent of 50:50 v/v of acetonitrile/methanol when using a water matrix. A validation batch was prepared in soil using this modification, but the improved recovery was not replicated.

A literature search was conducted of the published literature on terbufos and its analysis. It was found that there had been some observations of terbufos instability (mainly in water). Performing the extraction on ice, solved the poor recovery issue with the water matrix, therefore this modification was then used to prepare another soil batch (keeping the soil samples and reagent solutions on ice as much as possible, until required). However, the results still showed poor recovery for the terbufos analyte when using a soil matrix.

The sponsor contacted the original validation laboratory to discuss the observed continued poor recovery for the terbufos analyte, and how to further investigate the issue. It was agreed to perform an investigation to extract some test fortified soil samples at the original laboratory using an analyst that had not worked with the method previously, and after analysis at the original laboratory the extracts were re-injected at the independent validation laboratory. During the course of this investigation it was found that the time taken to perform the extraction was the critical factor (if the extraction procedure was interrupted in the middle or delayed in some way, this led to poor recovery of terbufos). If the extraction procedure (from fortification of the matrix to final dilution step) was carried out within a period of 2.5 hours, with no stoppages, good recovery was obtained for all three analytes.

The validation batches were then performed using this minor clarification of the original method (adding a time limit for the extraction procedure), which were all successful with acceptable recoveries of all three analytes.

3.2 Preparation of analytical standard solutions

3.2.1 Stock and fortification standard solutions

A weighed amount (corrected for purity if required) of the analytical standards were dissolved in acetonitrile to produce individual stock standard solutions of terbufos, terbufos sulfoxide and terbufos sulfone. An aliquot of the stock standard solution was taken to prepare a mixed secondary solution. The mixed secondary solution was progressively diluted to, 10 µg/mL and 1 µg/mL with acetonitrile to give fortification standard solutions.

3.2.2 Solvent-based instrument calibration solutions

The stock solution was 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.

3.3 Apparatus, glassware etc

Balances (various ranges)
Volumetric flasks (various sizes)
Volumetric pipettes (various sizes)
Polypropylene tubes (15 mL)
Pipettes (various sizes)
Oasis HLB SPE cartridge
Measuring cylinders (various sizes)

3.4 Preparation of reagents

Methanol:water (90:10 v:v)

methanol (900 mL) is mixed thoroughly with water (100 mL).

Acetonitrile:water (60:40 v:v)

acetonitrile (60 mL) is mixed thoroughly with water (40 mL).

Acetonitrile:water (20:80 v:v)

acetonitrile (20 mL) is mixed thoroughly with water (80 mL).

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.

Methanol:formic acid (100/0.1 v:v)

methanol (1000 mL) is mixed thoroughly with formic acid (1 mL).

3.5 Validation

Sub-samples of each type of untreated soil or sediment were fortified at known concentrations of the analytes (using mixed fortification solutions containing all three analytes), and analysed according to the following regime:

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

These samples were then processed using the analytical methodology described in Section 3.6.

3.6 Sample extraction procedure

1. Transfer a sub-sample of soil/sediment (20 g) to a 250 mL polyethylene bottle.
2. Add fortification solution at this stage if required.
3. Add methanol:water (90:10 v:v, 80 mL) and securely cap the bottle.
4. Place on a mechanical shaker and shake at approximately 200 rpm for approximately 30 minutes.
5. Centrifuge at approximately 3500 rpm for approximately 3 minutes, to separate the phases.
6. Decant the supernatant into a new 250 mL polyethylene bottle.
7. Repeat the extraction as steps 3 to 6, combining the extracts in the 250 mL bottle.
8. Dilute the extract to volume (200 mL) with methanol:water (90:10 v:v) and mix well.
9. Transfer an aliquot (2.5 mL) of the extract to a 15 mL polypropylene tube.
10. Add water (5 mL) and mix well.

SPE clean up

11. Condition the Oasis HLB SPE cartridge with acetonitrile (3 mL) and water (3 mL), discarding the eluate.
12. Load the extract from step 10 onto the SPE cartridge, discarding the eluate.
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.
14. Elute the SPE cartridge with an aliquot (3 mL) of acetonitrile, collecting in a 15 mL polypropylene tube.
15. Dilute the final extract to volume (5 mL) with water. Final matrix concentration ≡ 0.05 g sample matrix / mL final extract.
16. Perform any further dilutions using acetonitrile: water (60:40 v:v), as required.
17. Quantify the samples by the use of LC-MS/MS.

Method clarification

Note: The steps 2 to 15 need to be performed within a maximum period of 2.5 hours.

3.7 LC-MS/MS analysis

Instrument:	Sciex API 5000
Data management system:	Analyst 1.4.2
Ionisation mode:	Positive Ionspray
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 x 50 mm, 1.7 µm), or equivalent
Column temperature:	45°C
Sample temperature:	+4°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 time:	Terbufos: approx. 2.1 minutes Terbufos sulfoxide: approx. 1.2 minute Terbufos sulfone: approx. 1.2 minute
LOQ:	0.01 mg/kg
LOD:	0.1 ng/mL (≡ 0.002 mg/kg in sample matrix)

4. Calculation of results

Validation samples were quantified using the following equation:

$$\text{Residue found } (\mu g/L) = x \times \frac{1}{M} \times D$$

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 sandy loam soil fortified at 0.1 mg/kg.

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

$516266 = 118000x - 291$

where $y = 516266$
$m = 118000$
$c = -291$

$$\text{Therefore, concentration of Terbufos } (x) = \frac{516266 + 291}{118000} = 4.3776 \text{ ng/mL}$$

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

$$\text{Terbufos detected } (\mu g/L) = \frac{4.3776 \text{ ng/mL} \times 1}{0.05 \text{ g/mL}} = 87.55 \text{ ng/g} = 0.08755 \text{ mg/kg}$$

$$\text{Recovery } (\%) = \frac{0.08755 \text{ mg/kg} \times 100}{0.1 \text{ mg/kg}} = 87.55\%$$