2. Materials

2.1 Analytical standard – tribufos

Identity	*Tribufos technical*
Chemical name (IUPAC)	S,S,S-tributyl phosphorotrithioate
Structure	$(O=)P(S(CH_2)_3CH_3)_2(S(CH_2)_3CH_3)$ — $CH_3(CH_2)_3S$–P(=O)–$S(CH_2)_3CH_3$, $S(CH_2)_3CH_3$
Storage conditions	Ambient
Batch number	NX81AX0015
CAS number	78-48-8
Purity	98.2%
Supplier	Sponsor
Re-test date	September 2012

Certificate of Analysis is presented in Appendix 1.

2.2 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 of each of the three matrix types were fortified with known concentrations of the analyte 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 analyte under the typical storage conditions of the final extracts if they are not quantified immediately after preparation. Processed control extracts (prepared in acetonitrile), fortified with tribufos 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 tribufos at a concentration of 2 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 that time.

3.3 Matrix effects

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

3.4 Analytical method

Samples were extracted using an acetone/hexane mixture. Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), monitoring ion transitions *m/z* 315>169 (quantitation) and *m/z* 315>57 (confirmation).

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

3.5 Fortification/calibration solutions

A stock standard solution (1 mg/mL) of tribufos was prepared by dissolving an accurately weighed amount in a suitable volume of acetonitrile, correcting for purity as appropriate. This stock solution was further diluted with acetonitrile to produce fortification solutions at 10 µg/mL, 1 µg/mL, and 0.1 µg/mL concentrations.

The instrument calibration solutions, over the concentration range 0.025 ng/mL to 2 ng/mL, were prepared by serial dilution of the fortification solution in acetonitrile, as detailed below:

Standard solution used (ng/mL)	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100	0.2	10	2
100	0.15	10	1.5
100	0.1	10	1
100	0.08	10	0.8
100	0.04	10	0.4
1	2	10	0.2
1	1	10	0.1
1	0.5	10	0.05
1	0.25	10	0.025

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:

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

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

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

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

Example calculation of tribufos 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 10, Appendix 2.

Linear regression $y = \mathrm{m}\,x + \mathrm{c}$

$$1.65554e5 = 221308x + 1872.39$$

where $y = 1.65554e5$
$\mathrm{m} = 221308$
$\mathrm{c} = 1872.39$

Therefore, concentration of tribufos (x) $= \dfrac{1.65554e5 - 1872.39}{221308} = 0.7396$ ng/mL

Matrix concentration = 0.01 g matrix/mL final extract
Dilution factor = 1

Tribufos detected (mg/kg) $= \dfrac{0.7396 \text{ ng/mL} \times 1}{0.01 \text{g/mL}} = 73.96$ ng/g = 0.0740 mg/kg

Recovery (%) $= \dfrac{0.0740 \text{ mg/kg} \times 100}{0.1 \text{mg/kg}} = 74\%$

Appendix 3 Analytical Method

DETERMINATION OF TRIBUFOS IN SOIL AND SEDIMENT

1. General principle

Samples are extracted using an acetone/hexane mixture. 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 (100 mL)
Measuring cylinders (various sizes)
Filter papers (Whatman number 1 or equivalent)

3. Materials	Typical Grade (or equivalent)
Acetonitrile	HPLC
Ammonium formate	AR
Methanol	HPLC
Formic acid	AR
Water	HPLC
Hexane	Glass Distilled
Acetone	AR
Octanol	AR
Sodium sulphate (anhydrous)	AR

4. Preparation of reagents

Preparation of acetone:hexane (5:95 v:v) - acetone (50 mL) is mixed thoroughly with hexane (950 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

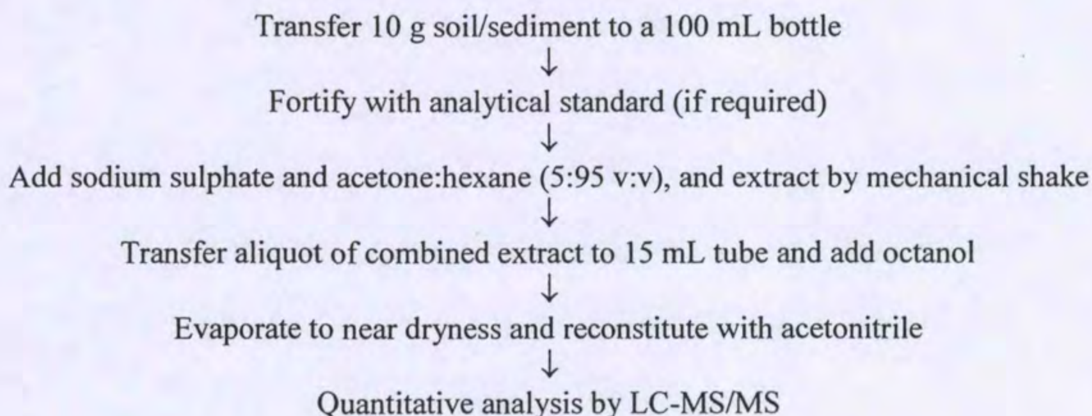
An appropriate amount of the test substance (corrected for purity) is accurately weighed and dissolved in acetonitrile to give the individual stock standard solution. Appropriate dilutions of the stock standard solution are made with acetonitrile to give mixed fortification standard solutions.

The mixed fortification solutions are progressively diluted with acetonitrile to produce a series of instrument calibration solutions in the range 0.025 to 2 ng/mL.

6. Procedure

6.1 Transfer a sub-sample of soil/sediment to a 100 mL polyethylene bottle.
6.2 Add fortification solution at this stage if required.
6.3 Add anhydrous sodium sulphate (approximately 5 g) and acetone:hexane (5:95 v:v, 45 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 through a funnel containing a Whatman Number 1 filter paper into a new 100 mL polyethylene bottle.
6.7 Repeat the extraction as steps 6.3 to 6.6, combining the extracts in the 100 mL bottle.
6.8 Dilute the extract to volume (100 mL) with acetone:hexane (5:95 v:v) and mix well.
6.9 Transfer an aliquot (1 mL) of the extract to a 15 mL polypropylene tube.
6.10 Add octanol (1 drop).
6.11 Evaporate to near dryness under nitrogen at approximately 30-40°C.
6.12 Reconstitute the sample extract in acetonitrile (10 mL), using ultrasonication to aid dissolution. Final matrix concentration ≡ 0.01 g soil/sediment / mL final extract.
6.13 Perform any further dilutions using acetonitrile, as required.
6.14 Quantify the samples by the use of LC-MS/MS.

7. Flow chart of analytical procedure



8. LC-MS/MS conditions

Instrument:	AB Sciex API 4000
Mode:	Ionspray positive
Ion monitoring details:	*m/z* 315>169 *m/z* 315>57 (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:	see table below
Cycle time:	4 min
Injection volume:	10 µL
Flow rate:	0.5 mL/min
Retention time:	approximately 1.5 minutes
LOQ:	0.01 mg/kg
LOD:	0.025 ng/mL (≡ 0.0025 mg/kg in sample matrix)

Gradient:

Time	%A	%B
0	30	70
0.2	30	70
2.0	5	95
2.5	5	95
3	30	70
4	30	70

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.