2 STUDY OBJECTIVE

The aim of this study was to validate the analytical method which is described in the Standard Operating Procedure CEM-3440/draft. This method has been developed at CEMAS for the determination of residues of Difenacoum in Sediment to a limit of quantification (LOQ) of 0.01 mg/kg. Validation was conducted on River Sediment.

3 MATERIALS / TEST SYSTEM

3.1 SPECIMENS

A specimen of river sediment was collected from Quelm Lane, Bracknell.

3.2 REFERENCE ITEM

Table 3: Reference items

Identity:	Difenacoum
Batch no:	01200609
Purity:	99.8%
Expiry date:	30 June 2012
Storage:	Ambient

The reference item will be retained until expiry and then disposed of. A copy of the Certificate of Analysis is given in Appendix 3.

4 EXPERIMENTAL PROCEDURES

4.1 FORTIFICATION OF SPECIMENS

Control specimens were fortified with Difenacoum as detailed below:

Matrix	Untreated Replicates	Replicates at Fortification Level mg/kg	
		LOQ	10 x LOQ
Sediment	2	5 at 0.01	5 at 0.1

4.2 METHOD OF ANALYSIS

Specimens were analysed using CEMAS SOP CEM-3440 (draft) 'Analytical Method for the Determination of Difenacoum in Sediment'.

The method originally developed and validated for the determination of difenacoum in sediment was based on extraction with chloroform (acetone/chloroform (50/50, v/v)). Following Sponsor review of the audited draft Final Report and audited draft SOP, it was requested that the method is modified so that chloroform is not used in the extraction process. A method check was carried out based on extraction with acetone/hexane (80/20, v/v). The SOP was modified and successfully validated. The data collected using acetone/chloroform (50/50, v/v) extraction solution is superseded and is not reported.

A copy of the finalised issued SOP, CEM-3440/001 is included in this report (Appendix 4).

Samples were extracted with acetone/hexane (80/20, v/v). After centrifugation an aliquot of the extract was purified on MAX SPE cartridges and eluted with ethyl acetate/methanol/formic acid (90/8/2, v/v/v). The samples were dried and re-dissolved in acetonitrile/water (80/20, v/v).

Quantitation was performed by the external standardisation with linearity.

The limit of quantitation (LOQ) for this method is 0.01 mg/kg.

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

CEMAS

SOP No. CEM-3440/001

1 INTRODUCTION

This SOP describes the procedures for the determination of difenacoum in sediment using LC-MS/MS.

2 SUMMARY

Residues are extracted from sediment extracted with acetone/hexane (80/20, v/v) followed by SPE extraction and final determination is by LC-MS/MS. The LOQ is 0.01 mg/kg difenacoum. At least two recoveries should be analysed with each batch. One of these will usually be fortified at the LOQ and the other at 10 times the LOQ or at a higher level if this is anticipated in the samples.

3 SAFETY PRECAUTIONS

Please refer to the relevant COSHH assessments and material safety data sheets (MSDS).

4 APPARATUS

- Centrifuge, available from Fisher Scientific
- 250 mL polypropylene bottles, available from Fisher Scientific
- 15 mL polypropylene centrifuge tubes, available from Fisher Scientific
- Gilson Microman pipettes and tips, available from Anachem
- Waters Oasis solid phase extraction cartridges, Oasis MAX 6 cc, 150 mg, part number 18600370, available from Waters
- 22 mL glass vials, available from Crawford Scientific
- Techne sample concentrator, available from Fisher Scientific
- HPLC autosampler vials, available from Crawford Scientific
- Luna 3µ Phenyl-Hexyl HPLC column (150 mm, 3.0 mm), part number 00F-4256-Y0, available from Phenomenex
- Agilent 1100 series Liquid Chromatography System
- Applied Biosystems MDS SCIEX API 4000 LC-MS/MS System

Note: Equivalent equipment may be substituted where appropriate.

5 REAGENTS AND SOLUTIONS

- Deionised water
- HPLC grade Acetone, A/0606/17, available from Fisher Scientific
- HPLC grade Hexane, H/0406/17, available from Fisher Scientific
- Ethyl acetate, residue grade, E/0903/17, available from Fisher Scientific
- HPLC Methanol, M/4056/17, available from Fisher Scientific
- HPLC grade water, W/0106/17, available from Fisher Scientific
- HPLC grade acetonitrile, A/0626/17, available from Fisher Scientific
- Formic acid, 98%, F/1900/PB08, available from Fisher Scientific
- Difenacoum reference material
- 35% Ammonia Solution, 0.88 S.G., A/3280/PB15, available from Fisher Scientific

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

- Ammonium Acetate, A/3440/53, available from Fisher Scientific
- 10mM Ammonium Acetate solution, 0.77 g ammonium acetate in 1 litre of HPLC Water
- 5% (v/v) Ammonia in Water, 5 mL 35% Ammonia Solution made up to 100 mL with Deionised Water.

Note: Reagents of equal high purity may be substituted where appropriate.

6 REFERENCE ITEMS FOR CALIBRATION AND FORTIFICATION

The preparation of these standard solutions may be achieved by the use of sensible serial dilutions. Alternate concentrations may be used as appropriate to the analysis. Solutions of reference items should be stored at approximately 4°C.

6.1 Fortification Standards

1000 µg/mL Standard Solution
Weigh accurately 100 mg of difenacoum into a 100 mL volumetric flask and make up to volume with acetone.

1.0 µg/mL Standard Solution
Serial dilute the 1000 µg/mL standard solution as appropriate in acetone.

0.1 µg/mL Standard Solution
Pipette 10 mL of the 1.0 µg/mL standard into a 100 mL volumetric flask and make up to volume with acetone.

6.2 Calibration Standards

1000 µg/mL Standard Solution
Weigh accurately 100 mg of difenacoum into a 100 mL volumetric flask and make up to volume with acetone.

10 µg/mL Standard Solution
Serial dilute the 1000 µg/mL standard solution as appropriate in acetone.

1.0 µg/mL Standard Solution
Serial dilute the 10 µg/mL standard solution as appropriate in acetonitrile/HPLC water (80/20).

0.1 µg/mL Standard Solution
Serial dilute the 1.0 µg/mL standard solution as appropriate in acetonitrile/HPLC water (80/20).

0.01 µg/mL Standard Solution
Serial dilute the 0.1 µg/mL standard solution as appropriate in acetonitrile/HPLC water (80/20).

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

CEMAS

SOP No. CEM-3440/001

7 SPECIMEN EXTRACTION AND CLEANUP

7.1 Controls and Reagent Blank

At least two unfortified control samples should be analysed with each set of samples. A reagent blank may also be included in a batch if deemed necessary.

7.2 Fortification of Samples

Appropriately fortified control samples should be analysed with each set of samples to assess the analytical efficiency of the method. Normally two recoveries will be analysed, one of these will usually be fortified at the LOQ and the other at 10 times the LOQ or at a higher level if this is anticipated in the samples.

7.3 Extraction

a) Weigh 10 g of matrix into a suitable extraction vessel *e.g.* a 250mL polypropylene bottle. Fortifications, if required, should be carried out at this point.
b) Add 100 mL of acetone/hexane (80/20 v/v).
c) Shake the sample on a shaker at a suitable speed, for example 150 rpm, for 30 minutes.
d) Centrifuge samples at a speed which visibly separates the solid sample from the supernatant, for example 3,500 rpm for 5 minutes.

7.3.1 Solid Phase Extraction Procedure

a) Transfer 10 mL of extract to a 15 mL polypropylene tube, add 100 µL of ammonia solution and shake well.
b) Take one 150 m g, 6 cc Waters Oasis MAX cartridge for each sample to be analysed.
   i. Wash step
   Add 5 mL of methanol to each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.
   ii. Conditioning step
   Add 5 mL of water to the each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.
   iii. Conditioning step
   Add 5 mL methanol to each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.
   iv. Sample loading
   Add the samples to the SPE cartridges and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

v. Wash step
Add 5 mL of ethyl acetate to each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.

vi. Wash step
Add 5 mL of methanol to each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.

vii. Wash step
Add 5 mL of 5% (v/v) ammonia in water solution to each cartridge and allow to pass through under gravity or under low vacuum, do not allow the cartridge to become dry.

viii. Dry the cartridge under vacuum for approximately 1 minute to remove any excess solvent before the elution step.

ix. Analyte elution
Place 22 mL glass vials beneath each cartridge. Elute the analyte with 15 mL formic acid/ethyl acetate/methanol (2/90/8 v/v/v) solution.

c) Concentrate the sample to dryness at up to 60°C under a gentle stream of air.

d) Re-constitute in 5 mL acetonitrile/water (80:20 v/v), vortex mix and transfer to an LC autosampler vial ready for analysis. If any particulate is seen in the final solution it may be necessary to filter through a 0.45 µm filter prior to analysis. (All samples should be diluted to within the calibration range with matrix control if required). The final sample concentration is 0.2 g/mL.

7.4 Preparation of Calibration Standard Solutions

No significant enhancement or suppression of detector response was observed in the presence of the sediment matrix used in the validation in this laboratory. The measured matrix effects were less than 10%.

Calibration standards can be prepared on a batch by batch basis. It is suggested to prepare them as follows:

Parent concentration. (µg/mL)	Volume taken. (mL)	Made to Final Volume with 80/20 acetonitrile/water.* (mL)	Final Standard concentration. µg/mL
1.0	0.05	1.0	0.05
1.0	0.02	1.0	0.02
0.1	0.1	1.0	0.01
0.1	0.05	1.0	0.005
0.1	0.02	1.0	0.002
0.01	0.1	1.0	0.001

* If it is deemed necessary to prepare matrix match calibration standards then the standards can be made to final volume with control matrix.

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CEMAS

SOP No. CEM-3440/001

8 INSTRUMENTATION AND OPERATING CONDITIONS

8.1 Instrumentation

Agilent 1100 series Liquid Chromatography System comprising of a binary pump, degasser, autosampler, column oven.
Applied Biosystems MDS SCIEX API 5000 LC-MS/MS System, valco switching valve.
Applied Biosystems MDS SCIEX Analyst Software version 1.4.2.

8.2 Chromatography Conditions

Column	:	Luna Phenyl-Hexyl (3 µm 150 mm, 3.0 mm)
Column Temperature	:	30°C
Injection Volume	:	20 µL
Mobile Phase	:	A: 10 mM ammonium acetate, B: HPLC grade acetonitrile

Mobile phase composition:

Total Time (min)	Flow Rate (µL/min)	% A	% B
0.0	400	80	20
0.5	400	80	20
5.0	400	10	90
7.5	400	10	90
7.6	400	80	20
9.0	400	80	20

Diverter valve positioning:

Time (min)	Position
0.0	B
5.5	A
9.9	B

A: to mass spectrometer
B: to waste
Note: check to confirm that the peak elutes within the specified time window. Approximate retention time of Difenacoum under these conditions is 6.0 minutes.

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

CEMAS

SOP No. CEM-3440/001

8.3 Mass Spectrometer Conditions

Period 1 Experiment 1:

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray (TIS)
Resolution Q1:	Unit
Resolution Q3:	Unit

Quantitation Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
443.10	135.20	150.00	-59.0	-20.0	-12.0

Confirmatory Transition:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP	EP
443.10	293.10	150.00	-74.0	-19.0	-10.0

All Transitions:

TEM:	450.0
CAD:	5.0
CUR:	25.0
GS1:	40.0
GS2:	25.0
IonSpray Voltage:	-4500.0
DP:	-220.0
Interface Heater:	On

Note: These settings and conditions may be instrument specific and can be adjusted for achieving acceptable chromatography and required sensitivity.

8.4 Analysis

- Using a calibration standard, inject replicate aliquots of an appropriate concentration to obtain a reproducible response before proceeding.
- Inject no more than four samples between appropriate calibration standards.

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SOP No. CEM-3440/001

9 CALCULATION OF RESULTS

Prepare an appropriate calibration curve by plotting peak area versus concentration expressed in µg/mL. Using appropriate regression analysis determine the equation of the line and the coefficient of determination for each analyte.

For example

If using a straight line equation, generate the following equation:

$y = mx + c$

Where x = concentration (µg/mL)
y = peak area
m = slope
c = intercept (c = 0 when the curve is forced through zero)

This equation may then be used to calculate the residues in individual specimens.

The extraction efficiency of procedural recovery specimens should be determined as follows;

$$\% \text{ Recovery} = \frac{(\text{Residue (mg/kg)}) - (\text{Apparent Residue in Control (mg/kg)})}{\text{Fortification Level (mg/kg)}} \times 100$$

10 METHOD VALIDATION

The method was validated for sediment in the CEMAS Study CEMS-4470.

10.1 Accuracy

Accuracy data was derived from the fortified specimens. The mean recovery value at each level and the overall mean recovery for each matrix was determined and was in the range 70% to 110% for difenacoum, indicating that the method shows good accuracy.

10.2 Precision

Precision data was derived from the fortified specimens. The RSD at each fortification level and the overall RSD for each matrix was no greater than 20% for difenacoum, indicating that the method shows good precision.

10.3 Linearity

The linear response of the instrumentation was evaluated for matrix-matched standards by making single determinations at 6 concentrations covering the range 0.001 µg/mL to 0.05 µg/mL for difenacoum. The coefficient of determination (r^2) was greater than or equal to 0.995 for all analytical determinations.

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CEMAS
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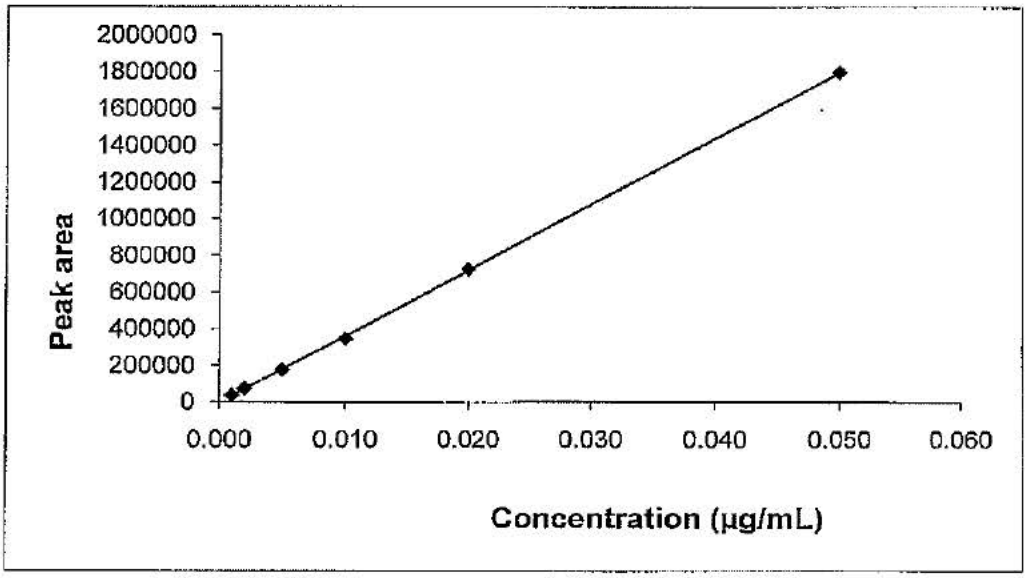
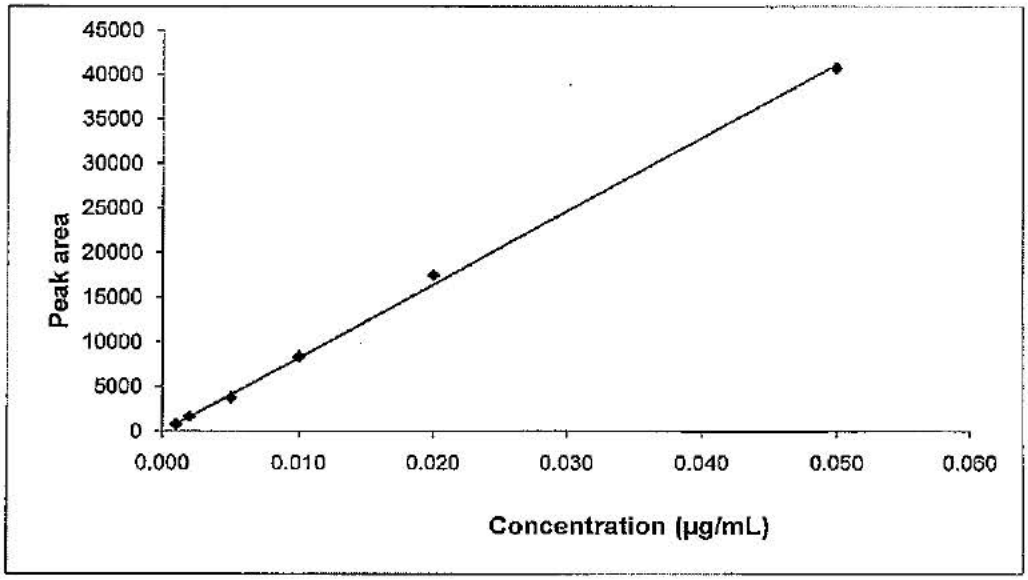
Figure 1: Linearity of the Detector Response for Difenacoum using Solvent Standards (Quantitation Transition, m/z 443.1 → 135.2)



Analyte Concentration (µg/mL)	Peak Area
0.05	1798182
0.02	724595
0.01	345099
0.005	177021
0.002	74147
0.001	35753

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

Figure 2: Linearity of the Detector Response for Difenacoum using Solvent Standards (Confirmatory Transition, m/z 443.1 → 293.1)



Analyte Concentration (µg/mL)	Peak Area
0.05	40767
0.02	17519
0.01	8379
0.005	3762
0.002	1656
0.001	786

APPENDIX 4: CEMAS SOP CEM-3440/001 CONTINUED

10.4 Specificity

The analytical method developed for the determination of difenacoum in sediment matrices has been shown to be highly specific due to the instrumentation used (LC-MS/MS) and the detection of two separate ion transitions.

10.5 Limit of Quantitation

The limit of quantification (LOQ) of the analytical method was confirmed as 0.01 mg/kg. The limit of quantitation is defined as the lowest fortification level at which acceptable recovery data are obtained. The mean recovery for specimens fortified at 0.01 mg/kg difenacoum was in the range 70 to 110% for sediment.

10.6 Limit of Detection

The limit of detection of the analytical method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample. An estimate of the LOD was taken as four times background noise. Note that the LOD may vary between runs and from instrument to instrument.

The Limit of detection (LOD) was estimated by using the following formula:

$$LOD = c_{standard} \frac{4xNoise}{h_{peak}},$$

where $c_{standard}$ is a concentration of the lowest standard, h_{peak} is the peak height and *Noise* is the background noise height. For this study the LOD was 0.020 ng/mL for difenacoum.

10.7 Method Confirmation

An additional confirmatory method was not necessary because LC-MS/MS is a highly specific method.