1 Summary

The purpose of the study was to carry out an independent laboratory validation (ILV) of the analytical method CAM-0004/003 for the determination of the phenoxy acids 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p (as the total phenoxy acid, when present in the matrix as the acid or ester) in drinking water to a limit of quantitation (LOQ) of 0.01 µg/L (0.02 µg/L for Mecoprop-p).

In brief, the method involves hydrolysis of samples overnight in a strong aqueous solution of sodium hydroxide to convert the ethyl-hexyl esters back to the parent acid for quantification. The hydrolysed samples are acidified then purified/concentrated using a solid phase extraction step prior to quantification by LC-MS/MS. Excerpts from the analytical method CAM-0004/003 and the actual method as used by Eurofins Agroscience Services Chem Ltd are included in Appendices C and D, respectively, as reference to the analytical procedure used for analysis.

The method was successfully validated at the second attempt for each analyte. At the first attempt residues of some analytes were detected above 30% of the LOQ in the tap water used as the control matrix. The validation was repeated using bottled mineral water an alternative source of control matrix, no residues above 30% of the LOQ were detected in this control matrix.

The ILV was carried out in two separate batches each consisting of a reagent blank, 2 control specimens, 5 recoveries fortified at the LOQ and 5 recoveries fortified at x10 LOQ (or x5 LOQ for Mecoprop-p). One batch was fortified with the acid and one batch was fortified with the corresponding 2 ethyl-hexyl ester. In each case residues were quantified as the parent acid residue. Where recoveries were fortified with the 2 ethyl-hexyl ester, they were fortified at an equimolar concentration to the intended acid concentration (acid equivalent).

2 Reference Items

Common name	2,4-D
Chemical name (IUPAC)	2,4-Dichlorophenoxyacetic acid
CAS-Registry-No.	94-75-7
Nufarm Standard No.	S1189
Batch reference	SP547-55
Purity	99.98 % (w/w)
Validation Date	20 Dec 2011
Re-test Date	19 Dec 2017
Storage conditions at test facility	Ambient

Common name	2,4-D 2EH
Chemical name (IUPAC)	2,4-Dichlorophenoxyacetic acid, 2-ethylhexyl ester
CAS-Registry-No.	1928-43-4
Nufarm Standard No.	S1248
Batch reference	S1198
Purity	99.19 % (w/w)
Validation Date	14 Feb 2013
Re-test Date	13 Feb 2015
Storage conditions at test facility	Refrigerated

Common name	2,4-DB
Chemical name (IUPAC)	4-(2,4-Dichlorophenoxy)butyric acid
CAS-Registry-No.	94-82-6
Nufarm Standard No.	S1271
Batch reference	RPH/566/95
Purity	99.93 % (w/w)
Validation Date	31 May 2013
Re-test Date	30 May 2019
Storage conditions at test facility	Ambient

Copies of their certificates of analysis are included in Appendix E.

2 Reference Items (continued)

Common name	2,4-DB 2EH
Chemical name (IUPAC)	4-(2,4-Dichlorophenoxy)butyric acid, 2-ethylhexyl ester
CAS-Registry-No.	7720-36-7
Nufarm Standard No.	S1295
Batch reference	S1167
Purity	99.51% (w/w)
Validation Date	22 Aug 2013
Re-test Date	21 Aug 2015
Storage conditions at test facility	Refrigerated

Common name	MCPA
Chemical name (IUPAC)	4-Chloro-2-methylphenoxyacetic acid
CAS-Registry-No.	94-74-6
Nufarm Standard No.	S1270
Batch reference	CN/572/48
Purity	99.28% (w/w)
Validation Date	03 May 2013
Re-test Date	02 May 2017
Storage conditions at test facility	Ambient

Common name	MCPA 2EH
Chemical name (IUPAC)	4-Chloro-2-methylphenoxyacetic acid, 2-ethylhexyl ester
CAS-Registry-No.	29450-45-1
Nufarm Standard No.	S1258
Batch reference	S1210
Purity	99.45% (w/w)
Validation Date	18 Apr 2013
Re-test Date	17 Apr 2015
Storage conditions at test facility	Refrigerated

Copies of their certificates of analysis are included in Appendix E.

2 Reference Items (continued)

Common name	MCPB
Chemical name (IUPAC)	4-(4-Chloro-2-methylphenoxy)butyric acid
CAS-Registry-No.	94-81-5
Nufarm Standard No.	S1286
Batch reference	S1163
Purity	99.77% (w/w)
Validation Date	26 Jul 2013
Re-test Date	25 Jul 2017
Storage conditions at test facility	Ambient

Common name	MCPB 2EH
Chemical name (IUPAC)	4-(4-Chloro-2-methylphenoxy)butyric acid, 2-ethylhexy ester
CAS-Registry-No.	94232-74-3
Nufarm Standard No.	S1318
Batch reference	S1188
Purity	95.18% (w/w)
Validation Date	18 Nov 2013
Re-test Date	17 Nov 2015
Storage conditions at test facility	Refrigerated

Common name	Mecoprop-p, (CMPP-p)
Chemical name (IUPAC)	(R+) 2-(4-chloro-2-methylphenoxy)propionic acid
CAS-Registry-No.	16484-77-8
Nufarm Standard No.	S1142
Batch reference	DC/532/22
Purity as Mecoprop	99.87% (w/w)
Purity as Mecoprop-p	99.71% (w/w)
Validation Date	31 May 2011
Re-test Date	30 May 2015
Storage conditions at test facility	Ambient

Copies of their certificates of analysis are included in Appendix E.

2 Reference Items (continued)

Common name	Mecoprop-p 2EH
Chemical name (IUPAC)	(R+)-2-(4-chloro-2-methylphenoxy)propionic acid, 2-ethylhexy ester
CAS-Registry-No.	861229-15-4
Nufarm Standard No.	S1296
Batch reference	S1178
Purity as Mecoprop-p 2EH	99.49% (w/w)
Purity as Mecoprop 2EH	99.58% (w/w)
Validation Date	22 Aug 2013
Re-test Date	21 Aug 2015
Storage conditions at test facility	Refrigerated

Common name	Dichloroprop-p, (2,4-DP-p)
Chemical name (IUPAC)	(R+) 2-(2,4-dichlorophenoxy)propionic acid
CAS-Registry-No.	15165-67-0
Nufarm Standard No.	S1211
Sample Identification	SP547-96
Purity as Dichloroprop	99.98% (w/w)
Purity as Dichloroprop-p	99.98% (w/w)
Validation Date	07 May 2012
Re-test Date	06 May 2016
Storage conditions at test facility	Ambient

Common name	2,4-DP-p 2EH
Chemical name (IUPAC)	(R+) 2-(2,4-Dichlorophenoxy)propionic acid, 2-ethylhexyl ester
CAS-Registry-No.	865363-39-9
Nufarm Standard No.	S1259
Sample Identification	S1209
Purity as 2,4-DP-p 2EH	99.86% (w/w)
Purity as 2,4-DP 2EH	99.86% (w/w)
Validation Date	24 Apr 2013
Re-test Date	23 Apr 2015
Storage conditions at test facility	Refrigerated

Copies of their certificates of analysis are included in Appendix E.

3 Internal Standards

Common name	2,4,6-TMAA (internal standard)
Chemical name (IUPAC)	(2,4,6-Trimethyl-Phenoxy)acetic acid
Batch reference	0001522273
Storage conditions at test facility	Ambient

Common name	4-CDMAA (internal standard)
Chemical name (IUPAC)	(4-Chloro-3,5-Dimethyl-Phenoxy) acetic acid
Batch reference	0001522274
Storage conditions at test facility	Ambient

Internal standards were purchased from Sigma-Aldrich, no certificate of analysis was provided. This is not deemed to be an issue for the purposes of what the internal standards are used for. Purity was assumed to be 100 % and an expiry date of 1 year after receipt was assigned to each.

4 Test System

4.1 Specimen Origin

Still mineral water was used as control matrix in this study and was purchased from a local supermarket.

4.2 Specimen Preparation

No preparation was necessary for drinking water.

4.3 Fortification Levels

The ILV was carried out in two separate batches each consisting of a reagent blank, 2 control specimens, 5 recoveries fortified at the LOQ and 5 recoveries fortified at x10 LOQ (or x5 LOQ for Mecoprop-p). One batch was fortified with the acid and one batch was fortified with the corresponding 2 ethyl-hexyl ester. In each case residues were quantified as the parent acid residue. Where recoveries were fortified with the 2 ethyl-hexyl ester, they were fortified at an equimolar concentration to the intended acid concentration (acid equivalent).

4.4 Test Method

The method CAM-0004/003 entitled 'Analytical method for the determination of phenoxy acids and their corresponding 2 ethyl-hexyl esters and glycine conjugates in cereal grain, straw and foliage, bovine muscle, fat, liver, kidney and milk, poultry eggs, citrus fruit and olives and phenoxy acids and their corresponding 2 ethyl-hexyl esters in surface water, soil and air' was validated for the quantification of 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p residues in drinking water. Sections 4 to 12 of the analytical method CAM-0004/003 are included in Appendix C as reference to the analytical method used for analysis and the actual method used by Eurofins Agroscience Services Chem Ltd., is included in Appendix D.

In brief, the method involves hydrolysis of samples overnight in a strong aqueous solution of sodium hydroxide to convert the ethyl-hexyl esters back to the parent acid for quantification. The hydrolysed samples are acidified then purified/concentrated using a solid phase extraction step prior to quantification by LC-MS/MS.

The method was successfully validated at the second attempt for each analyte. At the first attempt residues above 30% LOQ were detected in the control matrix for some analytes and these results have not been reported. The validation was repeated using an alternative source of control matrix.

5.5 Quantification

An appropriate calibration curve was prepared by plotting the peak area ratio (analyte peak area / internal standard peak area) versus concentration ratio (analyte concentration / internal standard concentration) (ng/mL). Using 1/x weighted linear regression, the equation of the line and correlation coefficient was determined.

The following equation was obtained:

$y = mx + c$

Where x = concentration (ng/mL)
y = peak area ratio
m = slope
c = intercept

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

$$\text{Residue } (\mu g/L) = \frac{\text{Residue in Final Volume (ng/mL)}}{\text{Sample Concentration (mL/mL)*}}$$

$$\text{Residue in Final Volume (ng/mL)} = \frac{(\text{Peak Area Ratio} - \text{Intercept})}{\text{Slope}}$$

$$\text{Peak Area Ratio} = \frac{\text{Peak Area of Analyte}}{\text{Peak Area of Internal Standard}}$$

*Initial sample volume (100 mL) / Final extract volume (0.5 mL)

Appendix C Excerpts from the Analytical Method CAM-0004/003

Used for study S14-01199. A.I. CEMAS 22.9.14

22.9.14

CEMAS

CEMAS ANALYTICAL METHOD

METHOD No. CAM-0004/003

TITLE ANALYTICAL METHOD FOR THE DETERMINATION OF PHENOXY ACIDS AND THEIR CORRESPONDING 2 ETHYL-HEXYL ESTERS AND GLYCINE CONJUGATES IN CEREAL GRAIN, STRAW AND FOLIAGE, BOVINE MUSCLE, FAT, LIVER, KIDNEY AND MILK, POULTRY EGGS, CITRUS FRUIT AND OLIVES AND PHENOXY ACIDS AND THEIR CORRESPONDING 2 ETHYL-HEXYL ESTERS IN SURFACE WATER, SOIL AND AIR

Lisa Allen
Author

11 August 2014
Effective Date

Mandy Griffiths
QA Review

11 August 2014
Date

Alan Whittle
Technical Review

11 August 2014
Date

DATA REQUIREMENTS

ENV/JM/MONO(2007)17
SANCO/825/00 rev 8.1 (2010)
SANCO/3029/99 rev 4 (2000)
OPPTS 860.1340 (1996)
OCSPP 850.6100 (2012)

REVISIONS TO PREVIOUS VERSIONS

Version	Reason for Re-issue
CAM-0004/002	Addition of matrices: bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit, olives.
CAM-0004/003	Addition of matrices: surface water, soil, air.

CEMAS
METHOD No. CAM-0004/003

1 INTRODUCTION

1.1 Objective

The purpose was to validate this analytical method, CAM-0004/003, for the analysis of the phenoxy acids 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-P and Dichloroprop-P (as the total phenoxy acid, when present in the matrix as the acid or ester) in water, soil and air and (as the total phenoxy acid, when present in the matrix as the acid, ester or conjugate) in cereal grain, straw, foliage, bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit and olives. The limit of quantitation (LOQ) is 0.01 mg/kg for cereal grain, straw, foliage, bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit, olives and soil, 0.05 µg/tube for air and 0.01 µg/L for surface water (for 2,4-D, 2,4-DB, MCPA, MCPB and Dichloroprop-P) and 0.02 µg/L for surface water (for Mecoprop-P).

This analytical method was validated under the CEMAS GLP Studies CEMS-6228 for cereal grain, straw and foliage, CEMS-6229 for bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit and olives and CEMS-6230 for surface water, soil and air.

The validation studies CEMS-6228, CEMS-6229 and CEMS-6230 were conducted to fulfil the data requirements outlined in the U.S EPA Residue Chemistry Guideline OPPTS 860.1340, the European Commission Guidance document on Residue Analytical Methods SANCO/825/00 rev 8.1 and the European Commission Guidance document on Generating and Reporting Methods of Analysis SANCO/3029/99 rev. 4.

1.2 Summary

This analytical method describes the procedure for the determination of the total phenoxy acid present in cereal grain, straw, foliage, bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit and olives, whether in the form of the acid, ester (eg. ethyl-hexyl) or conjugate (eg. glycine) and the total phenoxy acid present in surface water, soil and air, whether in the form of the acid or ester (eg. ethyl-hexyl).

During the extraction procedure samples are hydrolysed to convert the ethyl-hexyl esters and glycine conjugates back to the parent acid for quantitation.

The analysis is performed using a hydrolysis reaction, QuEChERS extraction and determination by LC-MS/MS detection. The LOQ for all analytes in cereal grain, straw, foliage, bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit, olives and soil is 0.01 mg/kg, the LOQ for all analytes in air is 0.05 µg/tube, the LOQ for 2,4-D, 2,4-DB, MCPA, MCPB and Dichloroprop-P in surface water is 0.01 µg/L and the LOQ for Mecoprop-P in surface water is 0.02 µg/L.

Samples are hydrolysed overnight in a strong aqueous solution of sodium hydroxide to convert the ethyl-hexyl esters and glycine conjugates back to the parent acid for quantitation. The hydrolysed samples are acidified and, with the exception of the water extraction procedure where QuEChERS is not required, analytes extracted into acetonitrile using QuEChERS before being concentrated for analysis. The reverse phase LC-MS/MS setup uses a monolithic column and flow split to optimise sensitivity.

This analytical method was successfully validated to a limit of quantitation (LOQ) of 0.01 mg/kg for all analytes in cereal grain, straw, foliage, bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit, olives and soil, an LOQ of 0.05 µg/tube for all analytes in air, an LOQ of 0.01 µg/mL for 2,4-D, 2,4-DB, MCPA, MCPB and Dichloroprop-P in surface water and an LOQ of 0.02 µg/mL for Mecoprop-P in surface water under the CEMAS GLP Studies CEMS-6228 (for cereal grain, straw and foliage),

CEMS-6229 (for bovine muscle, fat, liver, kidney, milk, poultry eggs, citrus fruit and olives) and CEMS-6230 (for surface water, soil and air). All of the validation criteria set out in the guidelines were met.

2 GLP COMPLIANCE

No GLP compliance is claimed for this analytical method. This method has been validated under the CEMAS GLP Studies CEMS-6228, CEMS-6229 and CEMS-6230.

3 SAFETY PRECAUTIONS

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

4 MATERIALS AND EQUIPMENT

Recommended equipment and consumables are listed in Appendix 1. Equivalent equipment and consumables may be substituted where appropriate. The reagents used in this method are listed in Appendix 2. Reagents of equal purity may be substituted where appropriate.

5 REFERENCE ITEMS FOR CALIBRATION AND FORTIFICATION

The preparation of standards or analyte mixtures can be achieved by serial dilution. Alternate concentrations may be used as appropriate to the analysis. It is recommended that separate stock solutions for calibration standards and fortification standards are prepared and that all reference items are stored at 4°C in amber 40 mL glass vials. The specific solvents used for achieving dilution are different based upon analyte solubility characteristics, and are indicated in the sections below.

Note that this method does not distinguish between the optical isomers of either Mecoprop-P or Dichloroprop-P, hence results obtained using this method are the sum of the optical isomers of Mecoprop or Dichloroprop as appropriate. The method does distinguish between Mecoprop and Dichloroprop, and each may be determined in the presence of the other.

Note that the 2-ethyl-hexyl ester and glycine conjugate analytes are intended to be fully hydrolysed back to the corresponding phenoxy acid for quantitation and are therefore only required during the method validation stage in order to demonstrate successful hydrolysis. **Routine use of the method does not require preparation of ester or conjugate solutions.** Since the recovery efficiencies of all forms of the molecule are comparable, separate validation of each form is not required in routine situations.

5.1 Stock solutions

5.1.1 1000 µg/mL Stock Solutions of Phenoxy acids (2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-P and Dichloroprop-P)

Prepare individual stock solutions by accurately weighing e.g. 20.00 mg of each reference item (corrected for purity) into a glass boat, transfer to a 20 mL volumetric flask and dilute to volume with acetonitrile. Once the item is fully dissolved transfer the contents to an amber glass vial/bottle for storage.

5.1.2 1000 µg/mL Stock Solutions of Phenoxy acid 2-Ethyl-hexyl Esters (2,4-D 2-EH, 2,4-DB 2-EH, MCPA 2-EH, MCPB 2-EH, Mecoprop-P 2-EH and Dichloroprop-P 2-EH) – For method validation only. Not required for routine use of the method.

Prepare individual stock solutions by accurately weighing e.g. 20.00 mg of each reference item (corrected for purity) into a glass boat, transfer to a 20 mL volumetric flask and dilute to volume with acetonitrile. Once the reference item is fully dissolved transfer the contents to an amber glass vial/bottle for storage.

5.1.3 1000 µg/mL Stock Solutions of Phenoxy acid Glycine Conjugates (2,4-D Glycine, 2,4-DB Glycine, MCPA Glycine, MCPB Glycine, Mecoprop-P Glycine and Dichloroprop-P Glycine) – For method validation only. Not required for routine use of the method.

Prepare individual stock solutions by accurately weighing e.g. 20.00 mg of each reference item (corrected for purity) into a glass boat, transfer to a 20 mL volumetric flask and dilute to volume with acetonitrile/water (50/50, v/v). Once the reference item is fully dissolved transfer the contents to an amber glass vial/bottle for storage.

5.1.4 1000 µg/mL Stock Solutions of Internal Standards ((2,4,6-trimethyl phenoxy)-acetic acid and (4-chloro-3,5-dimethylphenoxy)-acetic acid)

Prepare individual stock solutions by accurately weighing e.g. 10.00 mg of each reference item (corrected for purity) into a glass boat, transfer to a 10 mL volumetric flask and dilute to volume with methanol/water (50/50, v/v). Once the reference item is fully dissolved transfer the contents to an amber glass vial/bottle for storage.

5.2 Secondary Reference Items

Separate stock solutions should be used for the preparation of fortification and calibration standards. All standards should be stored in amber glass vials/bottles.

5.2.1 Phenoxy acids

10 µg/mL Solution of Phenoxy acids

Transfer 0.25 mL of each stock solution required, into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

1 µg/mL Solution of Phenoxy acids

Transfer 2.5 mL of the 10 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

0.1 µg/mL Solution of Phenoxy acids

Transfer 2.5 mL of the 1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

0.01 µg/mL Solution of Phenoxy acids (only required for water analysis)

Transfer 2.5 mL of the 0.1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

5.2.2 Phenoxy acid 2 Ethyl-hexyl Esters

The 2 ethyl-hexyl ester analytes are intended to be fully hydrolysed back to the corresponding acid and are therefore only required during the method validation stage to demonstrate successful hydrolysis. Solutions should be prepared at an equimolar concentration to the intended acid concentration (acid equivalent).

10 µg/mL Solution of Phenoxy acid esters (acid equivalent)

The following volumes of ester stock solution should be added to a 25 mL volumetric flask and made to volume with acetonitrile*.

2,4-D 2-EH	= 377 µL
2,4-DB 2-EH	= 364 µL
MCPA 2-EH	= 391 µL
MCPB 2-EH	= 374 µL
Mecoprop-P 2-EH	= 382 µL
Dichloroprop-P 2-EH	= 371 µL

*based on addition of 250µL which is corrected for the ratio of the molecular weight of the ester:acid

1 µg/mL Solution of Phenoxy acid esters (acid equivalent)

Transfer 2.5 mL of the 10 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile.

0.1 µg/mL Solution of Phenoxy acid esters (acid equivalent)

Transfer 2.5 mL of the 1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile.

0.01 µg/mL Solution of Phenoxy acid esters (acid equivalent) (only required for water analysis)

Transfer 2.5 mL of the 0.1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile.

5.2.3 Phenoxy acid Glycine Conjugates

The glycine conjugates are intended to be fully hydrolysed back to the corresponding acid and are therefore are only required during the method validation stage to demonstrate successful hydrolysis. Solutions should be prepared at an equimolar concentration to the intended acid concentration (acid equivalent).

10 µg/mL Solution of Phenoxy acid glycine conjugates (acid equivalent)

The following volumes of glycine conjugate stock solution should be added to a 25 mL volumetric flask and made to volume with acetonitrile/water (50/50, v/v)*.

2,4-D Glycine = 315 µL
2,4-DB Glycine = 308 µL
MCPA Glycine = 321 µL
MCPB Glycine = 312 µL
Mecoprop-P Glycine = 317 µL
Dichloroprop-P Glycine = 311 µL

*based on addition of 250µL which is corrected for the ratio of the molecular weight of the glycine conjugate:acid

1 µg/mL Solution of Phenoxy acid glycine conjugates (acid equivalent)

Transfer 2.5 mL of the 10 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

0.1 µg/mL Solution of Phenoxy acid glycine conjugates (acid equivalent)

Transfer 2.5 mL of the 1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile/water (50/50, v/v).

0.01 µg/mL Solution of Phenoxy acid esters (acid equivalent) (only required for water analysis)

Transfer 2.5 mL of the 0.1 µg/mL solution into a 25 mL volumetric flask and make to volume with acetonitrile.

5.2.4 Internal Standards

The two internal standards, (2,4,6-trimethyl-phenoxy)acetic acid (2,4,6-TMAA) and (4-chloro-3,5-dimethylphenoxy)acetic acid (4-CDMAA) , are used for quantitation of the phenoxy acids. See the table below listing which internal standard is used to quantitate each analyte.

Analyte	Internal Standard
2,4-D	2,4,6-TMAA
2,4-DB	4-CDMAA
MCPA	4-CDMAA
MCPB	2,4,6-TMAA
Mecoprop-P	4-CDMAA
Dichloroprop-P	4-CDMAA

5 µg/mL Internal Standard Solution Mixture

Pipette 0.5 mL of each of the 1000 µg/mL stock solutions (from section 5.1.4) for (2,4,6-trimethyl-phenoxy)acetic acid and (4-chloro-3,5-dimethylphenoxy)acetic acid into a 100 mL volumetric flask and make to volume with methanol/water (50/50, v/v). The resulting concentrations of the internal standards are 5 µg/mL respectively, ready to be used for analysis.

6 SPECIMEN EXTRACTION & CLEANUP FOR CEREAL MATRICES

6.1 Specimen Preparation

Where appropriate, specimens are prepared by homogenisation with dry ice.

6.2 Controls and Reagent Blank

At least one unfortified control sample should be analysed with each set of samples. A reagent blank may also be included in a batch if deemed necessary. It is recommended that additional controls are taken through the extraction procedure for preparation of matrix-matched standards.

6.3 Fortification of Samples

Appropriately fortified control samples should be analysed with each batch of samples to assess the analytical efficiency of the method. Normally two recovery levels will be analysed, one fortified at the LOQ and one fortified at a higher level. See the table below for suitable fortification procedures. The total fortified solution volume should not exceed 0.2 mL.

Matrices	Recovery Level (mg/kg)	Specimen Weight (g)	Reference Item Concentration (µg/mL)	Fortification Volume (mL)	Sample Concentration (g/mL)	Final Sample 'Expected Concentration' (µg/mL)
Cereal grain Cereal straw Cereal foliage	0.01	2	0.1	0.2	0.2	0.002
	0.1	2	1.0	0.2	0.2	0.02

6.4 Extraction procedure (cereals only)

a) Weigh 2 g of cereal matrix into a 40 mL glass vial with PTFE lids.
b) Fortify the procedural recovery samples as described in the table above.
c) Add 20 mL (10 mL*) of sodium hydroxide hydrolysis solution (47% sodium hydroxide/ deionised water, **15/85**, v/v) and 1 mL methanol, ensuring that the samples are fully wetted (this may require stirring with a spatula). Cap the tubes securely and mix thoroughly by hand.
d) Place the samples in a heater block or an oven overnight (≥ 16 hours) at 85°C to hydrolyse. The hydrolysed samples are feculent and have an unpleasant odour so the following four steps should be carried in a fume cupboard.
e) Allow the samples to cool until the contents are lukewarm, and then add 5 mL (2.5 mL*) of chilled 15N sulphuric acid to lower the pH to approximately 3. Shake vigorously by hand to mix thoroughly (release gas pressure in tube by loosening lid slightly after initial shaking).
f) Transfer the contents to a 50 mL centrifuge tube and then rinse remaining contents of glass vial into the centrifuge tube with 4 mL (2 mL*) of the chaotropic reagent 1M monochloroacetic acid and vortex mix.
g) Add 9 mL of acetonitrile to the centrifuge tube and vortex mix.
h) To each tube add the QuEChERS salts (EN 15662) using a wide bore funnel, cap the tube and shake vigorously to agitate and dissolve the salt agglomerates. Then add 10 mL of hexane and shake vigorously for a few seconds.
i) When all samples have been completed place the tube rack (horizontally) on a flat-bed shaker to continue mixing for another 30 minutes.

j). Centrifuge the tubes for 15 minutes at 3500rpm, 4°C. Pre-cool the centrifuge if necessary to reach the desired temperature.
k) Take a 1 mL aliquot of the middle solvent (acetonitrile/methanol) layer and transfer to a 96-deep well plate (2.2 mL volume) (alternatively HPLC vials can be used).
l) Evaporate the samples to incipient dryness under nitrogen at 40°C (a few µL's remaining or no longer than 5 minutes drying whilst no solvent remains). Reconstitute the samples in 1 mL of HPLC water + 0.2 % formic acid /acetonitrile (60/40, v/v).
m) Prepare the matrix-matched standards as shown in section 6.5. Then add 10 µL of internal standard solution to all matrix-matched standards and all samples.
n) Cap the plate and mix well on an orbital shaker for 15 minutes, avoiding cross well spillage (if using HPLC vials cap and mix by hand).
o) The final sample concentration is 0.2 g/mL. The phenoxy herbicides are analysed together, as indicated in the LC-MS/MS methodology described in Section 11.

* Alternative reagent volumes for use with high moisture content cereal matrices. See Section 6.7.

6.5 Preparation of Matrix-Matched Batch Calibration Standards

It is recommended that matrix-matched calibration standards are prepared within a 96-well plate (or alternatively HPLC vials) on the day of analysis. Extract three control samples using the QuEChERS procedure (Section 6.4 a-j), enough to provide 10 individual acetonitrile aliquots (1 blank and 9 equivalent matrix blanks for standard preparation) (Section 6.4 k). An example of the standards and spiking scheme is shown below, but the concentration range can be adjusted accordingly to fit with expected concentration levels in the samples:

Standard Mixture Concentration (µg/mL)	Vol. of Standard mixture added (µL)	Volume of control extract added (µL)	Final standard concentration (ng/mL)
0.1	6	994	0.6
0.1	15	985	1.5
0.1	30	970	3
0.1	50	950	5
1	10	990	10
1	20	980	20
1	50	950	50
10	10	990	100
10	20	980	200

The acetonitrile aliquots should be taken through the subsequent drying process (Section 6.4 l) for preparation at step 'm'.

6.6 Assay Time

A typical analytical batch of approximately 20 samples can be completed by one person over 2 working days (approximately 3 hours on the first day and approximately 7.5 hours on the second day). Samples should be left at the hydrolysis stage overnight (step 6.4 d.) between the first and second day. It is not advised to stop the extraction overnight at any other stage, although acceptable procedural recoveries will validate any work flow interruptions.

6.7 Experimental Precautions

In the event of high procedural recoveries, consideration should be given to the moisture content of the cereal matrix being used. For matrices with high moisture content, use the *alternative reagent volumes highlighted in the extraction procedure (stages 6.4 c, e, f).* High moisture content can cause incomplete phase separation between the organic and aqueous phases during the QuEChERS extraction, leading to <10 mL volume of the middle solvent layer being seen at Stage 6.4 k resulting in high recovery.

HPLC grade ultra-pure water, when used to prepare the LC mobile phase, results in less background noise in the MS/MS chromatography than water taken from most laboratory water purification systems.

CEMAS
METHOD No. CAM-0004/003

8 SPECIMEN EXTRACTION & CLEANUP FOR WATER

8.1 Specimen Preparation

No preparation necessary. Water should be allowed to reach room temperature prior to extraction.

8.2 Controls and Reagent Blank

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

8.3 Fortification of Samples

Appropriately fortified control samples should be analysed with each batch of samples to assess the analytical efficiency of the method. Normally two recovery levels will be analysed, one fortified at the LOQ and one fortified at a higher level. See the table below for suitable fortification procedures. The total fortified solution volume should not exceed 0.5 mL.

Matrix	Recovery Level (µg/L)	Specimen Volume (mL)	Reference Item Concentration (µg/mL)	Fortification Volume (mL)	Sample Concentration (L/mL)	Final Sample 'Expected Concentration' (µg/mL)
Surface Water (for 2,4-D, 2,4-DB, MCPA, MCPB and Dichloroprop-P)	0.01	100	0.01	0.1	0.2	0.002
	0.1	100	0.1	0.1	0.2	0.02
Surface Water (for Mecoprop-P)	0.02	100	0.01	0.2	0.2	0.004
	0.1	100	0.1	0.1	0.2	0.02

8.4 Extraction procedure (water only)

a) Transfer 100 mL of water to a 100 mL glass bottle.
b) Fortify the procedural recovery samples as required.
c) Add 1 mL of sodium hydroxide hydrolysis solution (47% sodium hydroxide/ deionised water, 15/85 v/v) and mix gently by hand.
d) Place the samples in an oven at 85°C overnight (≥ 16 hours) to hydrolyse.
e) Allow the samples to cool and add 1 mL of 15N sulphuric acid and mix gently by hand.
f) Condition a Strata X (30 mg / 3 mL) SPE cartridge with 3 mL of methanol followed by 3 mL of 0.05% hydrochloric acid in water.
g) Load entire acidified water sample onto an SPE cartridge using reservoirs.
h) Wash SPE cartridge with 3 mL of methanol/water/hydrochloric acid 40/60/0.5 and 3 mL of deionised water.
i) Elute SPE cartridge with 2 x 2 mL of 1% ammonia in acetonitrile.
j) Evaporate to dryness and reconstitute in 0.5 mL of injection buffer (60/40 0.2% formic acid in water/acetonitrile).
k) Prepare batch standards as described in section 8.5.

l) Add 5 µL of internal standard solution to all standards and samples.
m) Cap plate or vials and mix gently.
n) The final sample concentration is 0.2 L/mL. The phenoxyherbicides are analysed together, as indicated in the LC-MS/MS methodology in section 11.

8.5 Preparation of Non Matrix-Matched Batch Calibration Standards

Standard Mixture Concentration (µg/mL)	Vol of Standard mixture added (µL)	Volume of injection buffer (µL)	Final sample concentration (ng/mL)
0.1	3	497	0.6
0.1	7.5	492.5	1.5
0.1	15	485	3
0.1	25	475	5
1	5	495	10
1	10	490	20
1	25	475	50
10	5	495	100
10	10	490	200

8.6 Assay Time

A typical analytical batch of approximately 20 samples can be completed by one person over 2 working days (approximately 3 hours on the first day and approximately 5 hours on the second day). Samples should be left at the hydrolysis stage overnight (step 8.4 d.) between the first and second day. It is not advised to stop the extraction overnight at any other stage, although acceptable procedural recoveries will validate any work flow interruptions. It is recommended that LC-MS/MS analysis be carried out as soon as possible after extraction.

8.7 Experimental Precautions

It is recommended to test control samples prior to use, to ensure they contain suitably low background levels of the relevant analytes.

HPLC grade ultra-pure water, when used to prepare the LC mobile phase, results in less background noise in the MS/MS chromatography than water taken from most laboratory water purification systems.

CEMAS
METHOD No. CAM-0004/003

11 INSTRUMENTATION & OPERATING CONDITIONS

The method has been developed for use on a Symbiosis Pharma LC system (LC only) linked to an Applied Biosystems Sciex API4000 triple quadrupole mass spectrometer. Other instrumentation can also be used, though optimization may be required to achieve the desired separation and sensitivity.

Final determination by LC-MS/MS with 2 characteristic isotopic mass transitions is considered to be highly specific and no further confirmatory conditions are included.

Typical Liquid Chromatography Operating Conditions

Instrumentation:	Symbiosis Pharma Liquid Chromatography System AB Sciex 4000 triple quad MS System AB Sciex Analyst 1.4.2 data system
Column:	Onyx C18 monolithic column, 3.0 x 100 mm
Guard Column: (if required)	Chromolith RP-18 end capped guard cartridge 5 x 3 mm
Column Temperature:	Ambient
Injection Volume:	40 µL
Run Time:	Approx. 9 minutes
Mobile Phase:	A: HPLC water + 0.1 % formic acid B: Methanol + 0.1 % formic acid
Flow:	1 mL/min, split 1:4 to the mass spectrometer

Gradient:

Time (min:secs)	A %	B %
0:01	55	45
0:03	55	45
6:00	25	75
6:01	5	95
7:15	5	95
7:16	55	45
9:00	55	45

Flow Program: Diverter
1) 0.0 → 1.8 min: flow to waste
2) 1.8 → 6.5 min: flow to source
3) 6.5 → end of run: flow to waste

CEMAS
METHOD No. CAM-0004/003

Typical Mass Spectrometry Operating Conditions

Ionization mode:	ESI
Probe Position:	8 mm
Period 1 (2.6 minutes)	
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	25
Collision Gas (CAD):	10
Temperature (TEM):	300°C
GS1:	30
GS2:	40
Ion Spray Voltage:	-4500 V
Dwell time (msec):	75
Entrance Potential:	-10

Compound:	Q1 Mass (amu)	Q3 Mass (amu)	DP	CE	CXP	Internal standard	Retention Time (min)
2,4-D Quantitation	218.8	161.0	-40	-18	-8	2,4,6-TMAA	3.43
2,4-D Confirmation	220.8	162.9	-40	-18	-8	2,4,6-TMAA	3.43
MCPB Quantitation	227.0	140.9	-60	-20	-12	2,4,6-TMAA	5.21
MCPB Confirmation	229.0	142.9	-60	-20	-12	2,4,6-TMAA	5.21
MCPA Quantitation	199.0	140.9	-40	-20	-8	4-CDMAA	3.65
MCPA Confirmation	200.9	142.9	-40	-20	-8	4-CDMAA	3.65
Mecoprop-P Quantitation	212.9	140.9	-40	-18	-8	4-CDMAA	4.55
Mecoprop-P Confirmation	215.0	142.9	-40	-18	-8	4-CDMAA	4.55
Dichloroprop-P Quantitation	232.9	160.8	-40	-16	-8	4-CDMAA	4.45
Dichloroprop-P Confirmation	234.9	162.8	-40	-16	-8	4-CDMAA	4.45
2,4-DB Quantitation	247.0	161.0	-30	-18	-9	4-CDMAA	5.10
2,4-DB Confirmation	249.0	163.0	-40	-18	-9	4-CDMAA	5.10
2,4,6-TMAA (IS1)	193.0	135.0	-40	-18	-9	n/a	4.11
4-CDMAA (IS2)	212.9	155.0	-40	-20	-9	n/a	4.43

CEMAS
METHOD No. CAM-0004/003

12 ANALYSIS & CALCULATION OF RESULTS

- Using a calibration standard inject replicate aliquots of an appropriate concentration to obtain a reproducible response before proceeding.
- Bracket samples with calibration standards. The calibration should have a minimum of 5 points and all samples should be within the calibration range.
- Inject no more than four samples between calibration standards.
- Insert blank injections between samples of high concentration or before controls to eliminate the effect of column carryover.

The extraction recovery efficiency of fortified samples should be determined as follows from the calculated values.

Prepare an appropriate calibration curve by plotting peak area versus concentration expressed in ng/mL. Using appropriate regression analysis (i.e. linear regression, 1/x weighted linear regression or other appropriate regression), determine the equation of the line and the correlation coefficient for each analyte.

For example:

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

$y = mx + c$

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

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

$$\text{Residue in Final Volume (µg/mL)} = \frac{(\text{Peak Area Ratio - Intercept})}{\text{Slope}} / 1000$$

$$\text{Residue (mg/kg)} = \frac{\text{Residue in Final Volume (µg/mL)}}{\text{Sample Concentration (g/mL)}}$$

$$\text{Peak Area Ratio} = \frac{\text{Peak Area of Analyte}}{\text{Peak Area of Internal Standard}}$$

Appendix D Actual method as used by Eurofins Agroscience Services Chem Ltd

A. Scope of the Method

The method has been successfully used in the determination of 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p in drinking water.

B. Analyte Information

Common name	2,4-D
Chemical name (IUPAC)	2,4-Dichlorophenoxyacetic acid
CAS-Registry-No.	94-75-7

Common name	2,4-D 2EH
Chemical name (IUPAC)	2,4-Dichlorophenoxyacetic acid, 2-ethylhexyl ester
CAS-Registry-No.	1928-43-4

Common name	2,4-DB
Chemical name (IUPAC)	4-(2,4-Dichlorophenoxy)butyric acid
CAS-Registry-No.	94-82-6

Common name	2,4-DB 2EH
Chemical name (IUPAC)	4-(2,4-Dichlorophenoxy)butyric acid, 2-ethylhexyl ester
CAS-Registry-No.	7720-36-7

Common name	MCPA
Chemical name (IUPAC)	4-Chloro-2-methylphenoxyacetic acid
CAS-Registry-No.	94-74-6

Common name	MCPA 2EH
Chemical name (IUPAC)	4-Chloro-2-methylphenoxyacetic acid, 2-ethylhexyl ester
CAS-Registry-No.	29450-45-1

Common name	MCPB
Chemical name (IUPAC)	4-(4-Chloro-2-methylphenoxy)butyric acid
CAS-Registry-No.	94-81-5

Common name	MCPB 2EH
Chemical name (IUPAC)	4-(4-Chloro-2-methylphenoxy)butyric acid, 2-ethylhexy ester
CAS-Registry-No.	94232-74-3

Common name	Mecoprop-p, (CMPP-p)
Chemical name (IUPAC)	(R+) 2-(4-chloro-2-methylphenoxy)propionic acid
CAS-Registry-No.	16484-77-8

Common name	Mecoprop-p 2EH
Chemical name (IUPAC)	(R+)-2-(4-chloro-2-methylphenoxy)propionic acid, 2-ethylhexy ester
CAS-Registry-No.	861229-15-4

Common name	Dichloroprop-p, (2,4-DP-p)
Chemical name (IUPAC)	(R+) 2-(2,4-dichlorophenoxy)propionic acid
CAS-Registry-No.	15165-67-0

Common name	2,4-DP-p 2EH
Chemical name (IUPAC)	(R+) 2-(2,4-Dichlorophenoxy)propionic acid, 2-ethylhexyl ester
CAS-Registry-No.	865363-39-9

Common name	2,4,6-TMAA (internal standard)
Chemical name (IUPAC)	(2,4,6-Trimethyl-Phenoxy)acetic acid

Common name	4-CDMAA (internal standard)
Chemical name (IUPAC)	(4-Chloro-3,5-Dimethyl-Phenoxy) acetic acid

C. Method Summary

Extraction	Overnight hydrolysis in a strong aqueous solution of sodium hydroxide
Clean up	Purification by SPE
Detection	Liquid chromatography with tandem mass spectrometry (LC-MS/MS)
Limit of Quantification	0.01 µg/L for 2,4-D, 2,4-DB, MCPA, MCPB and Dichloroprop-p 0.02 µg/L for Mecoprop-p
Reference	The method is based on the procedures described in the analytical method CAM-0004/003 as supplied by CEMAS.

D. Reagents and Materials

Information pertaining to the identity and source of reagents typically used is summarised in Table I. Alternative, equivalent reagents and materials may be used, unless specifically stated otherwise.

Table I. Identification of Reagents and Materials

- Acetonitrile
  (HPLC grade, Fisher Scientific)
- Acetonitrile
  (HiPerSolv Chromanorm LC/MS grade, Fisher Scientific)
- Ammonia Solution
  (S.G., 35%, Fisher Scientific)
- Formic acid
  (LC/MS Optima grade, Fisher Scientific)
- Formic acid
  (98+%, Fisher Scientific)
- Hydrochloric acid
  (37% certified AR, Fisher Scientific)
- Methanol
  (HiPerSolv Chromanorm LC/MS Grade, Fisher Scientific)
- Methanol
  (HPLC Grade, Fisher Scientific)
- Sodium hydroxide pellets
  (Sigma-Aldrich)
- Sulphuric acid
  (95%, SLR, extra pure, Fisher Scientific)
- Water
  (HPLC grade, Fisher Scientific)
- Water
  (HiPerSolv Chromanorm LC/MS Grade, Fisher Scientific)
- SPE cartridge
  (Strata X (30 mg / 3 mL), Phenomenex)

E. Instrumentation and Apparatus

Information pertaining to the identity of instruments and apparatus typically used is summarised in Table II. Alternative, equivalent instrumentation and apparatus may be used, unless specifically stated otherwise.

Table II. Identification of Instrumentation and Apparatus

- Common laboratory glassware
- Dri-block Heater
  (Techne DB-3, Fisher Scientific)
- Oven
  (Leader Engineering, UK)
- Ultrasonic bath
  (Ultrawave, Fisher Scientific)
- LC-MS/MS System
  (AB Sciex 5500 QTrap with an Agilent 1200 series HPLC pump / oven and CTC autosampler)

F. Mobile Phases

Mobile Phase A: 0.1 % formic acid in water

Measure approximately 900 mL of water in a graduated measuring cylinder and pipet 1 mL of formic acid (100 %) into the same cylinder. Bring to a final 1000 mL volume with water and mix well.

Mobile Phase B: 0.1 % formic acid in methanol

Measure approximately 900 mL of methanol in a graduated measuring cylinder and pipet 1 mL of formic acid (100 %) into the same cylinder. Bring to a final 1000 mL volume with methanol and mix well.

G. Preparation of Standard Solutions

Stock solutions of the analytes are prepared by dissolving a weight of the reference items with the aid of an ultrasonic bath. Each stock solution is allocated a unique reference number.

The stock solutions are further diluted for use as fortification solutions in the procedural recovery process and for subsequent use as solvent calibration solutions.

All solutions are typically stored 1 °C to 10 °C in a glass vial under dark conditions.

A summary of the typical dilutions to be carried out is presented in the following tables.

Table III. Preparation of a Stock Solution of 2,4-D in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100	20.01	20.01	20	1001	2,4-D

* to be taken from the Certificate of Analysis

Table IV. Preparation of a Stock Solution of 2,4-D 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.2	19.94	19.94	20	997	2,4-D2EH

* to be taken from the Certificate of Analysis

Table V. Preparation of a Stock Solution of 2,4-DB in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.9	20.00	20.00	20	1000	2,4-DB

* to be taken from the Certificate of Analysis

Table VI. Preparation of a Stock Solution of 2,4-DB 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.5	20.08	20.08	20	1004	2,4-DB2EH

* to be taken from the Certificate of Analysis

Table VII. Preparation of a Stock Solution of MCPA in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.3	20.02	20.02	20	1001	MCPA

* to be taken from the Certificate of Analysis

Table VIII. Preparation of a Stock Solution of MCPA 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.5	19.92	19.92	20	996	MCPA2EH

* to be taken from the Certificate of Analysis

Table IX. Preparation of a Stock Solution of MCPB in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.77	20.00	20.00	20	1000	MCPB1

* to be taken from the Certificate of Analysis

Table X. Preparation of a Stock Solution of MCPB 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
95.2	21.05	20.04	20	1002	MCPB2EH

* to be taken from the Certificate of Analysis

Table XI. Preparation of a Stock Solution of Mecoprop-p in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.87	20.01	20.01	20	1001	MECOP1

* to be taken from the Certificate of Analysis

Table XII. Preparation of a Stock Solution of Mecoprop-p 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
99.5	19.94	19.94	20	997	MEC2EH

* to be taken from the Certificate of Analysis

Table XIII. Preparation of a Stock Solution of Dichloroprop-p in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100	20.02	20.02	20	1001	DPP2,4-DP

* to be taken from the Certificate of Analysis

Table XIV. Preparation of a Stock Solution of Dichloroprop-p 2EH in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100	19.96	19.96	20	998	2,4-DPPEH

* to be taken from the Certificate of Analysis

Table XV. Preparation of a Stock Solution of 2,4,6-TMAA internal standard in Methanol/Water (50/50, v/v)

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100 (Assumed)	9.98	9.98	10	998	TMAAIS1

* to be taken from the Certificate of Analysis

Table XVI. Preparation of a Stock Solution of 4-CDMAA internal standard in Methanol/Water (50/50, v/v)

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100 (Assumed)	10.00	10.00	10	1000	CDMAAIS1

* to be taken from the Certificate of Analysis

Table XVII. Preparation of Mixed Phenoxy Acid Fortification Solutions of 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p in Acetonitrile/Water (50/50, v/v)

Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
2,4-D 2,4-DB MCPA MCPB1 MECOP1 DPP2,4-DP	1001 1000 1001 1000 1001 1001	0.25 (each)	25	10	ACIDM1
ACIDM1	10	2.5	25	1.0	ACIDM2
ACIDM2	1.0	2.5	25	0.1	ACIDM3
ACIDM3	0.1	5.0	25	0.02	ACIDM4

Table XVIII. Preparation of Phenoxy Acid Fortification Solutions of Mecoprop-p in Acetonitrile/Water (50/50, v/v)

Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
MECOP1	1001	0.25	25	10	MECOP2
MECOP2	10	0.25	25	0.1	MECOP3
MECOP3	0.1	5.0	25	0.02	MECOP4

Table XIX. Preparation of Mixed Phenoxy Acid 2-Ethyl-hexyl Ester Fortification Solutions of 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p in Acetonitrile

Reference of standard solution used	Concentration (µg/mL)	Volume taken (µL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
2,4-D2EH 2,4-DB2EH MCPA2EH MCPB2EH MEC2EH 2,4-DPPEH	997 1004 996 1002 997 998	378.5 362.6 393 373.4 383.4 371.6	25	10	ESTERM1
ESTERM1	10	2.5	25	1.0	ESTERM2
ESTERM2	1.0	2.5	25	0.1	ESTERM3
ESTERM3	0.1	5.0	25	0.02	ESTERM4

Table XX. Preparation of Phenoxy Acid 2-Ethyl Ester Fortification Solutions of Mecoprop-p in Acetonitrile

Reference of standard solution used	Concentration (µg/mL)	Volume taken (µL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
MEC2EH	997	383.4	25	10	MEC2EH2
MEC2EH2	10	250	25	0.1	MEC2EH3
MEC2EH3	0.1	5000	25	0.02	MEC2EH4

Table XXI. Preparation of Mixed Internal Standard Solutions of 2,4,6-TMAA and 4-CDMAA in Methanol/Water (50/50, v/v)

Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
TMAAIS1 CDMAAIS1	998 1000	0.5 (each)	100	5.0	ISMIX1

Table XXII. Preparation of Mixed Phenoxy Acid Solvent Calibration Solutions of 2,4-D, 2,4-DB, MCPA, MCPB, Mecoprop-p and Dichloroprop-p in 0.2 % formic acid in water/acetonitrile (60/40, v/v)

Reference of standard solution used	Concentration (µg/mL)	Volume taken (µL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
ACIDM3	0.1	3	0.5	0.6	SS1
ACIDM3	0.1	7.5	0.5	1.5	SS2
ACIDM3	0.1	15	0.5	3	SS3
ACIDM3	0.1	25	0.5	5	SS4
ACIDM2	1.0	5	0.5	10	SS5
ACIDM2	1.0	10	0.5	20	SS6
ACIDM2	1.0	25	0.5	50	SS7
ACIDM1	10	5	0.5	100	SS8
ACIDM1	10	10	0.5	200	SS9

These standards were also used as calibration standards for the quantification of the Phenoxy Acid 2-Ethyl Ester fortified specimens.

H. Specimen Preparation

No preparation necessary. Water should be allowed to reach room temperature prior to extraction.

I. Sample Weight(s) and Fortifications

In general, control (untreated) specimens of drinking water are fortified prior to extraction with the fortification solutions as described below. The analytes are fortified jointly. Fortification, extraction and quantification of the phenoxy acids and the phenoxy acid 2-ethyl esters were performed as two separate batches.

Table XXIII. Summary of Sample Weights and Fortifications – Phenoxy acid only

Fortified analytes	Matrix	Sample volume (mL)	Reference of mixed fortification solution used	Concentration of fortification solution (µg/mL)	Volume of fortification solution added (mL)	Fortification level (each) (µg/L)
2,4-D 2,4-DB MCPA MCPB Mecoprop-p Dichloroprop-p	Drinking water	100	ACIDM4 and MECOP4	0.02 (each)	0.05	0.01 and 0.02 (mecoprop-p only)
		100	ACIDM3	0.1	0.1	0.1

Table XXIV. Summary of Sample Weights and Fortifications – Phenoxy acid 2-ethyl esters **only**

Fortified analytes	Matrix	Sample volume (mL)	Reference of mixed fortification solution used	Concentration of fortification solution (µg/mL)	Volume of fortification solution added (mL)	Fortification level (each) (µg/L)
2,4-D 2EH 2,4-DB 2EH MCPA 2EH MCPB 2EH Mecoprop-p 2EH Dichloroprop-p 2EH	Drinking water	100	ESTERM4 and MEC2EH4	0.02 (each)	0.05	0.01 and 0.02 (mecoprop-p only)
		100	ESTERM3	0.1	0.1	0.1

J. Sample Work-Up Procedure

Extraction

Extraction of water:

1) Each 100 mL mixed specimen of water is measured into a 100-mL glass bottle. If required procedural recoveries are then fortified.
2) 1 mL of sodium hydroxide hydrolysis solution is added and mixed gently by hand.
3) The specimen is placed in an oven at 85 °C overnight (≥ 16 hours) to hydrolyse.
4) 1 mL of 15N sulphuric acid is added to the cooled specimen and mixed gently by hand.

Extract Clean-up and Reconstitution for Analysis

5) A Strata X (30 mg /3 mL) SPE cartridge is conditioned with 3 mL of methanol followed by 3 mL of 0.05% hydrochloric acid in water
6) The entire acidified water sample from step 4 is added onto the SPE cartridge using reservoirs.
7) The SPE cartridge is washed with 3 mL of methanol/water/hydrochloric acid 40/60/0.5 v/v/v and 3 mL of deionised water.
8) The SPE cartridge is eluted with 2 x 2 mL of 1% ammonia in acetonitrile.
9) The extract is evaporated to dryness and reconstituted in 0.5 mL of injection buffer (0.2% formic acid in water/acetonitrile, 60/40 v/v).
10) 5 µL of mixed internal standard solution is added to all samples and calibration standards prior to quantification using LC-MS/MS.

No changes or modifications were made to the original method of extraction, as detailed in CAM-0004/003.

K. Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions used for quantification is included in the following table:

Table XXV. Summary of chromatographic and mass spectrometric conditions

Chromatographic conditions				
HPLC system	Agilent 1200 series HPLC pump/oven and CTC auto-sampler			
Column	Onyx C18 (100 x 3.0 mm, Phenomenex)			
Column oven temperature	30 °C			
Injection volume	20 µL			
Mobile phases	Eluent A: Water containing 0.1 % (v/v) formic acid Eluent B: Methanol containing 0.1 % (v/v) formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.00	55	45	1000
	0.05	55	45	1000
	6.00	25	75	1000
	6.02	5	95	1000
	7.25	5	95	1000
	7.27	55	45	1000
	9.50	55	45	1000
Divert valve	0.0 min to 1.5 min to A; 1.5 min to 8.0 min to B			
Retention times	approx. 3.7 min (2,4-D); approx. 5.4 min (2,4-DB); approx. 3.9 min (MCPA); approx.. 5.5 min (MCPB); approx. 4.8 min (Mecoprop-p), approx.. 4.7 min (Dichloroprop-p)			
Mass spectrometric conditions				
MS system	AB Sciex 5500 QTrap LC/MS-MS System			
Ionisation type	Electrospray ionization (ESI, Turbolon Spray)			
Polarity	Negative ion mode			
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)			
Capillary voltage (IS)	-4500 V		Ionspray turbo heater (TEM)	375 °C
Curtain gas (CUR)	30 (arbitrary units)		Gas flow 1 (GS1)	45 (arbitrary units)
Collision gas (CAD)	4 (arbitrary units)		Gas flow 2 (GS2)	45 (arbitrary units)

Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
2,4-D	219 → 161#	-44	-4.5	-18	-13	45
	221 → 163	-47	-4.5	-18	-14	45
2,4-DB	247 → 161#	-30	-4.5	-12	-13	45
	249 → 163	-30	-4.5	-12	-10	45
MCPA	199 → 141#	-45	-4.5	-18	-11	45
	201 → 143	-54	-4.5	-18	-13	45
MCPB	227 → 141#	-40	-4.5	-13	-11	45
	229 → 143	-35	-4.5	-13	-12	45
Mecoprop-p	213 → 141#	-57	-4.5	-18	-11	45
	215 → 143	-50	-4.5	-18	-13	45
Dichloroprop-p	233 → 161#	-45	-4.5	-16	-14	45
	235 → 163	-42	-4.5	-16	-8	45
2,4,6-TMAA (IS1)	193 → 135	-40	-4.5	-18	-9	45
4-CDMAA (IS2)	213 → 155	-40	-4.5	-20	-9	45

used for quantification but both of the mass transitions listed can be used for quantification

The table below clarifies which internal standard is used to quantitate each analyte:

Analyte	Internal Standard
2,4-D	2,4,6-TMAA
2,4-DB	4-CDMAA
MCPA	4-CDMAA
MCPB	2,4,6-TMAA
Mecoprop-p	4-CDMAA
Dichloroprop-p	4-CDMAA

Only optimization of instrumental parameters were made to the original detection method detailed in CAM-0004/003.

L. Special Precautions

None

M. Calculation of Results

Intersperse calibration standards at random throughout the chromatographic sequence, in accordance with EAS UK SOPs, bracketing injections with a calibration standard and confirm detector linearity over the calibration range of interest by constructing a calibration curve of peak area ratio (analyte peak area / internal standard peak area) versus concentration ratio (analyte concentration / internal standard concentration). Linear regression, linear regression with 1/x weighting, quadratic or power curve relationships should be used where appropriate. The correlation coefficient (R) must be greater or equal to 0.995.

From the calibration curve ($y = mx + c$) calculate analyte residues as follows:

A_{Conc} =	$\frac{y - c}{m}$
A_{Conc}	Concentration of analyte in final extract (ng/mL)
y =	$\frac{\text{Peak area of analyte}}{\text{Peak Area of Internal Standard}}$
y	Peak area of ratio
c	Intercept of calibration curve
m	Gradient of calibration curve
R =	$\frac{A_{Conc} \times V_{End} \times DF}{W_{Initial}}$
R	Analyte Residue (µg/L)
A_{Conc}	Concentration of analyte in final extract (ng/mL)
V_{End}	Volume of final extract = 0.5 mL
DF	Dilution factor (for no dilution = 1)
$W_{Initial}$	Initial volume of matrix = 100 mL

An example calculation for a 2,4-D procedural recovery sample fortified at 0.1 mg/kg is presented below:

Analyte Concentration (ng/mL) =	$\frac{(1738954/7585330)\ (y) - 0.00119\ (c)}{0.0119\ (m)}$ = 19.2 ng/mL (rounded)
Analyte Residue (µg/L) =	$\frac{19.2\ (A_{Conc}) \times 0.5\ (V_{End}) \times 1\ (DF)}{100\ (W_{Initial})}$ = 0.0959 µg/L (rounded)
Recovery Value (%) =	$\frac{0.0959\ \mu g/L\ \text{(Analyte Residue Found)}}{0.1\ \mu g/L\ \text{(Fortification Level)}} \times 100$ = 96 % (rounded)

N. Estimated Time Required for Analysis

Recommended samples per set	13 samples
Measuring of aliquots for analysis	1 hour
Extraction and clean-up	20 hours
LC-MS/MS instrument setup	1 hour
Analysis by LC-MS/MS (incl. injection of calibration solutions)	4 hours (unattended instrument-hours)
Data evaluation	3 hours
Total	29 hours

O. Method Flow Chart

Sample measuring
100 mL of water
⇩
Extraction
Add 1 mL of sodium hydroxide solution,
mix by hand
Place in oven at 85 °C overnight (≥ 16 hours) to hydrolyse
Allow to cool and add 1 mL 15N sulphuric acid
mix gently by hand
⇩
Clean-up
Condition a Strata X SPE cartridge with 3 mL of methanol and 3 mL of 0.05% hydrochloric acid in water
Load entire acidified water sample onto SPE
Wash SPE with 3 mL methanol/water/hydrochloric acid (40/60/0.5, v/v/v) and 3 mL deionised water
Elute SPE cartridge with 2 x 2 mL of 1% ammonia in acetonitrile
Evaporate to dryness and reconstitute in 0.5 mL of injection buffer
Add 5 µL of mixed internal standard to all standards and samples
⇩
Analyse final sample extracts by LC-MS/MS

P. Safety Information

Reagents	Acetonitrile	highly flammable, toxic by inhalation, in contact with skin and if swallowed, irritating to eyes, respiratory system and skin, risk of serious damage to eyes Wear suitable eye protection, gloves and protective clothing In case of contact with eyes, rinse immediately with plenty of water and seek medical advice Take off immediately all contaminated clothing In case of accident or if you feel unwell, seek medical advice immediately (show label where possible) Keep away from sources of ignition - No smoking Waste disposal - flammable waste solvent drum
	Formic acid	flammable liquid and vapour, causes severe skin burns and eye damage Wear suitable eye protection, gloves and protective clothing In case of contact with eyes, rinse cautiously with water for several minutes Remove contact lenses, if present and easy to do. Continue rinsing Immediately call a poison center or doctor Waste disposal – acid waste drum
	Hydrochloric acid (37 %)	Harmful liquid and vapour, Causes burns, irritating to respiratory system, risk of blindness Wear suitable eye protection, gloves and protective clothing In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. In case of contact with skin, apply skin-protective barrier cream. If inhaled, move to fresh air and give oxygen if short of breath Take off all contaminated clothing immediately In case of accident or if you feel unwell, seek medical advice immediately (show label where possible) Waste disposal - acid waste solvent drum
	Methanol	highly flammable, poisonous by inhalation and ingestion. Use suitable eye protection, gloves and protective clothing. Waste disposal - flammable waste solvent drum
	Sodium hydroxide pellets	Non-flammable solid Use suitable eye protection, gloves and protective clothing. Very hazardous in case of skin contact (corrosive, irritant, permeator), of eye contact (irritant, corrosive), of ingestion and of inhalation. In case of contact with skin and eyes, immediately flush skin and eyes with plenty of water for at least 15 minutes. In case of serious contact with skin, wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. If inhaled, move to fresh air and give oxygen if short of breath. Do not induce vomiting if ingested In case of accident or if you feel unwell, seek medical advice immediately (show label where possible)

	Sulphuric acid	Non-flammable liquid Use suitable eye protection, gloves and protective clothing. Very hazardous in case of skin contact (corrosive, irritant, permeator), of eye contact (irritant, corrosive), of ingestion and of inhalation. In case of contact with skin and eyes, immediately flush skin and eyes with plenty of water for at least 15 minutes. In case of serious contact with skin, wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. If inhaled, move to fresh air and give oxygen if short of breath. Do not induce vomiting if ingested In case of accident or if you feel unwell, seek medical advice immediately (show label where possible) Waste disposal - acid waste solvent drum
Others	see MSDSs for relevant information	