

1 Summary

Study Objective

The objective of the study was to independently validate the method No. CAM-0004/003 for the determination of six phenoxy acids and their corresponding 2 ethyl-hexyl esters (2EH) in soil according to the guidance document SANCO/825/00, rev. 8.1, with a limit of quantification (LOQ) of 0.01 mg/kg (acid equivalents).

Analytical Procedure

In brief, samples were fortified with six phenoxy acids and their corresponding esters. Samples of soil were then hydrolysed with an aqueous sodium hydroxide solution and subsequently was lowered to approx. pH 3 with sulphuric acid. Acetonitrile and a salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. Hexane was added and the extract was shaken again. After centrifugation an aliquot of the acetonitrile phase was cleaned by adding a mixture of the SPE sorbents magnesium sulphate, aluminium oxide and graphitised carbon black. After centrifugation the samples were diluted with 0.2 % formic acid in water.

Selectivity

Quantification was performed by use of LC-MS/MS detection. Two (2) mass transitions were evaluated per analysed substance in order to demonstrate that the method achieves a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or the control sample extracts, so that a high level of selectivity was demonstrated.

Matrix Effects

Matrix effects on the detection of analyte(s) in extracts of soil were found to be significant ($\geq 20\%$) for the phenoxy acids 2,4-DB, MCPB as well as Mecoprop-P and insignificant ($\leq 20\%$) for 2,4-D, MCPA and Dichloroprop-P. Matrix-matched standards were used for quantification of all analytes.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of eight (8) concentration levels ranging from 0.6 ng/mL to 200 ng/mL. This range corresponds to 0.003 mg/kg to 1 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample extract.

The calibration curves obtained for both mass transitions of all phenox acids were linear since correlation coefficients (R) were ≥ 0.995 . Linear regression was performed with 1/x-weighting.

Quantification

Quantification was performed by using linear regression with additional correction for bracketing standards. Phenoxy esters were quantified as their corresponding acids.

Prior to analysis an internal standard was added to all sample extracts and calibration standard solutions. Quantification was performed using the internal standard 2,4,6-TMAA for the phenoxy acids 2,4-D and MCPB, for all other analytes 4-CDMAA was used as internal standard.

Accuracy and Precision

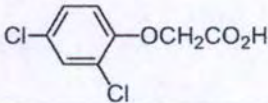
Accuracy was determined by fortification of control samples with known amounts of the test / reference item(s) and subsequent determination of the recoveries when applying the analytical method. Precision was

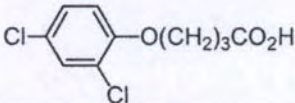
2 Study Objective

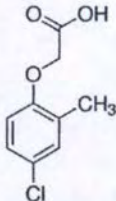
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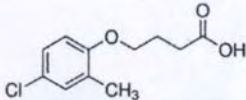
3 Materials and Methods

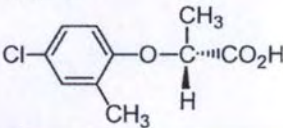
3.1 Test / Reference Item(s)

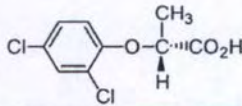
Test Item			
Test item name	2,4-D	Batch number	SP547-55
		Nufarm Standard No.	S1660
EAS Test item code	M-00014155	Appearance / colour	solid / white
Chemical name	2,4-dichlorophenoxyacetic acid		
CAS number	94-75-7	Purity analysed	99.98 % w/w
Chemical structure		Molecular weight	221.0 g/mol
Density	not applicable	Signal word(s)	danger
Issue date of certificate	07 Dec 2017	Expiry date	30 Nov 2023
		Storage conditions	ambient (≤ +30 °C), dark, dry

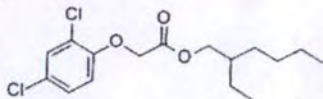
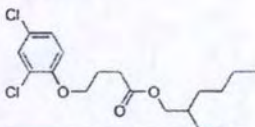
Test Item			
Test item name	2,4-DB	Nufarm Standard No.	S1271
EAS Test item code	M-00018655	Appearance / colour	solid / white
Chemical name	4-(2,4-dichlorophenoxy)butyric acid		
CAS number	94-82-6	Purity analysed	99.93 % w/w
Chemical structure		Molecular weight	249.1 g/mol
Relative density	1.461	Signal word(s)	warning
Issue date of certificate	03 Jun 2013	Expiry date	30 May 2019
		Storage conditions	ambient (≤ +30 °C), dark, dry

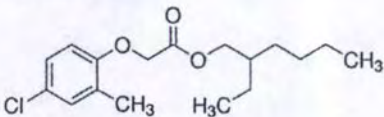
Test Item			
Test item name	MCPA	Nufarm Standard No.	S1472
EAS Test item code	M-00018654	Appearance / colour	solid / white
Chemical name	4-chloro-2-methylphenoxyacetic acid		
CAS number	94-74-6	Purity analysed	99.82 % w/w
Chemical structure		Molecular weight	200.6 g/mol
Density	not applicable	Signal word(s)	danger
Issue date of certificate	06 May 2016	Expiry date	21 Apr 2020
		Storage conditions	ambient ($\leq +30$ °C), dark, dry

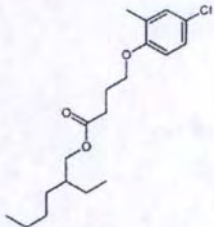
Test Item			
Test item name	MCPB	Nufarm Standard No.	S1477
EAS Test item code	M-00018657	Appearance / colour	solid / white
Chemical name	4-(4-chloro-2-methylphenoxy)butyric acid		
CAS number	94-81-5	Purity analysed	99.66 % w/w
Chemical structure		Molecular weight	228.7 g/mol
Density	not applicable	Signal word(s)	warning
Issue date of certificate	09 Jun 2016	Expiry date	08 Jun 2020
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

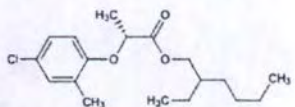
Test Item			
Test item name	Mecoprop-p	Nufarm Standard No.	S1631
EAS Test item code	M-00018648	Appearance / colour	solid / white
Chemical name	(R+)-2-(4-chloro-2-methylphenoxy)propionic acid		
CAS number	16484-77-8	Purity analysed	99.69 % w/w
Chemical structure		Molecular weight	214.6 g/mol
Density	not applicable	Signal word(s)	danger
Issue date of certificate	10 Oct 2017	Expiry date	29 Sep 2021
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

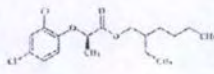
Test Item			
Test item name	Dichlorprop-p (2,4-DP-p)	Nufarm Standard No.	S1557
EAS Test item code	M-00018650	Appearance / colour	solid / white
Chemical name	R(+)-2-(2,4-dichlorophenoxy)propionic acid		
CAS number	15165-67-0	Purity analysed	99.56 % w/w
Chemical structure		Molecular weight	235.1 g/mol
Density	not applicable	Signal word(s)	danger
Issue date of certificate	01 Aug 2017	Expiry date	21 Jul 2021
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

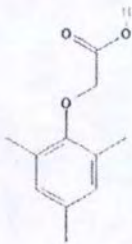
Test Item			
Test item name	2,4-D 2EH	Nufarm Standard No.	S1657
EAS Test item code	M-00018660	Appearance / colour	liquid / clear, colourless
Chemical name	2,4-dichlorophenoxyacetic acid, 2-ethylhexyl ester		
CAS number	1928-43-4	Purity analysed	99.58 % w/w
Chemical structure		Molecular weight	333.3 g/mol
Density	not available	Signal word(s)	warning
Issue date of certificate	15 Nov 2017	Expiry date	14 Nov 2019
		Storage conditions	cool (1 °C - 10 °C), dark, dry
Test Item			
Test item name	2,4-DB 2EH	Nufarm Standard No.	S1555
EAS Test item code	M-00018662	Appearance / colour	liquid / pale yellow
Chemical name	4-(2,4-dichlorophenoxy)butyric acid, 2-ethylhexyl ester		
CAS number	7720-36-7	Purity analysed	99.67 % w/w
Chemical structure		Molecular weight	361.3 g/mol
Relative density	1.119	Signal word(s)	warning
Issue date of certificate	01 Aug 2017	Expiry date	17 Jul 2019
		Storage conditions	cool (1 °C - 10 °C), dark, dry

Test Item			
Test item name	MCPA 2EH	Nufarm Standard No.	S1532
EAS Test item code	M-00018664	Appearance / colour	liquid / colourless
Chemical name	4-chloro-2-methylphenoxyacetic acid, 2-ethylhexyl ester		
CAS number	29450-45-1	Purity analysed	99.29 % w/w
Chemical structure		Molecular weight	312.8 g/mol
Relative density	1.074	Signal word(s)	danger
Issue date of certificate	30 Mar 2017	Expiry date	28 Mar 2019
		Storage conditions	cool (≤ +10 °C), dark, dry

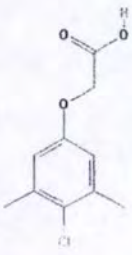
Test Item				
Test item name	MCPB 2EH	Nufarm Standard No.	S1695	
EAS Test item code	M-00018926	Appearance / colour	liquid / colourless	
Chemical name	4-(4-chloro-2-methylphenoxy)butyric acid, 2-ethylhexyl ester			
CAS number	94232-74-3	Purity analysed	96.23 % w/w	
Chemical structure			Molecular weight	340.9 g/mol
Density	not available	Signal word(s)	warning	
Issue date of certificate	27 Jul 2018	Expiry date	25 Jul 2020	
		Storage conditions	cool ($\leq +10$ °C), dark, dry	

Test Item				
Test item name	Mecoprop-p 2EH	Nufarm Standard No.	S1659	
EAS Test item code	M-00018652	Appearance / colour	liquid / clear, colourless	
Chemical name	R(+)-2-(4-chloro-2-methylphenoxy)propionic acid, 2-ethylhexyl ester			
CAS number	861229-15-4	Purity analysed	98.75 % w/w	
Chemical structure			Molecular weight	326.9 g/mol
Relative Density	1.042	Signal word(s)	warning	
Issue date of certificate	30 Nov 2017	Expiry date	27 Nov 2019	
		Storage conditions	cool (1 °C - 10 °C), dark, dry	

Test Item			
Test item name	Dichlorprop-P 2EH	Nufarm Standard No.	S1516
EAS Test item code	M-00018658	Appearance / colour	liquid / clear colourless
Chemical name	R(+)-2-(2,4-dichlorophenoxy)propionic acid, 2-ethylhexyl ester		
CAS number	865363-39-9	Purity analysed	98.96 % w/w
Chemical structure			
Relative density	1.1215	Signal word(s)	warning
Issue date of certificate	27 Jan 2017	Expiry date	25 Jan 2019
		Storage conditions	cool (1 °C - 10 °C), dark, dry

Test Item (used as internal standard)			
Test item name	(2,4,6-trimethyl-phenoxy)-acetic acid*	Batch number	B0714
EAS Test item code	M-00014154	Nufarm Standard No.	S1336
		Appearance / colour	solid / white
CAS number	13333-81-8	Purity analysed	100 area %
Chemical structure			
		Molecular weight	194.2 g/mol
Density	not applicable	Signal word(s)	danger
Issue date of certificate	not available	Expiry date	04 Oct 2018 *
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

*abbreviated as 2,4,6-TMAA

Test Item (used as internal standard)			
Test item name	(4-chloro-3,5-dimethylphenoxy)acetic acid*	Batch number	B0514
EAS Test item code	M-00014152	Nufarm Standard No.	S1335
CAS number	19545-95-0	Appearance / colour	solid / white
Chemical structure		Purity analysed	> 95 % w/w
		Molecular weight	214.7 g/mol
Density	not applicable	Signal word(s)	not available
Issue date of certificate	not available	Expiry date	04 Oct 2018 *
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

*abbreviated as 4-CDMAA

Specifications essential for correct identification of the test / reference item(s) and for use under GLP are based on the information as provided by the study sponsor (e.g. certificate(s) of analysis). They have not been verified by the test facility and might have not been generated under GLP.

Additional specifications for test / reference item characterisation originate from (non-GLP) sources other than the study sponsor.

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

3.2 Test System(s), Sample Origin, Preparation and Storage

Test System (Commodity)	Preparation	Origin
One Soil of sandy loam type	The soil was obtained in North Dakota and characterised at the test facility. No moisture content was determined. Residues are reported as wet soil	North Dakota, USA

Weighed sub-samples were stored at typically ≤ -18 °C in the dark until fortification and extraction.

Soil characterisation data are shown in Appendix F.

3.3 Method Summary

Method Reference(s)	Given by validation CAM-0004/0003 [1]
Extraction	Hydrolysis with aqueous sodium hydroxide solution
Clean up	Purification with QuEChERS salts and subsequently with dispersive SPE sorbents
Storage	Final sample extracts will be stored at 1 °C to 10 °C (target) in the dark until analysis
Detection	Liquid chromatography with tandem mass spectrometry (LC-MS/MS)
Limit of Quantification (LOQ)	0.01 mg/kg for all analytes
Limit of Detection	Calculated by signal/noise ratio, but at least below 30 % of the LOQ

The full method description including flow chart is given in Appendix A.

No addition or modification to the original method other than optimisation of instrumental parameters and no communication with the method developers or others familiar with the method was necessary in order to carry out the analysis.

I. Reagents and Materials

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

Table I: Identification of Reagents and Materials

- | |
|---|
| <ul style="list-style-type: none"> • Acetonitrile HPLC grade
(VWR, Art. No. 83639.320) • Methanol LCMS grade
(Honeywell, Art. No. 34966) • Water HPLC grade
(Merck, Art. No. 1.15333.2500) • Demineralized water
(prepared at laboratory) • n-Hexane for pesticide residue analysis
(VWR, Art. No. 83661.320) • Methanol
(Sigma Aldrich, Art. No. 34860-7L-R) • Sulphuric Acid 95-97 %
(VWR, Art. No. 20700.265) • Sodium Hydroxide
(VWR, Art. No. 28244.364) • Chloracetic Acid ≥ 99 %
(Sigma Aldrich, Art. No. 402923) • Citrat-Kit-01 (4g MgSO₄, 1g NaCl, 1g trisodiumcitrate, 0.5g disodium citrate sequihydrate)
(Bekolut, Art. No. CK-01-050) • Quechers Kit-33 (100mg MgSO₄, 50mg graphitised carbon black, 100mg aluminium oxide)
(Bekolut, Art. No. QUE-KIT-33) |
|---|

II. Instrumentation and Apparatus

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

Table II: Identification of Instrumentation and Apparatus

- Adjustable pipettes
(Eppendorf: Research 10-100 μL , Research 100-1000 μL , Research 0.5-10 μL , Brand: Transferpipette S 1000-10000 μL , Transferpipette S 500-5000 μL)
- Common laboratory glassware
- Balances
(Sartorius TE 2101, Sartorius CPA 224S)
- Vortex mixer
(Scientific Industries Genie 2)
- Flatbed Shaker
(Bühler Flatbed Shaker SM 30 A)
- Centrifuge
(Hettich Centrifuge Rotina 380, VWR Centrifuge Galaxy MiniStar)
- Drying Oven
(VWR DRY-Line drying cabinet DL 115)
- LC-MS/MS
(API 6500 triple quadrupole mass spectrometer, 1290 Infinity HPLC system)

III. Reagent Solutions and Mobile Phases

Water + 0.2 % formic acid

Measure approximately 100 mL of water in a graduated measuring cylinder and transfer the measured volume into an appropriate vessel. Add 200 μ L of formic acid and mix well.

47 % NaOH solution

Weigh 94 g of NaOH into an appropriate vessel and add 200 mL of water using a graduated measuring cylinder. Mix well.

47 % NaOH solution / water (15:85, v:v)

Measure approximately 425 mL of water and 75 mL of 47 % NaOH solution in separate graduated measuring cylinders. Transfer the measured volumes into an appropriate vessel and mix well.

15 N sulphuric acid

Measure approximately 74 mL of water and 51 mL of concentrated sulphuric acid in separate graduated measuring cylinders. Transfer the measured volumes into an appropriate vessel and mix well.

1 M Chloracetic acid

Weigh 18.9 g of chloracetic acid into an appropriate vessel and add 200 mL of water using a graduated measuring cylinder. Mix well.

Water + 0.2 % formic acid / acetonitrile (6:4, v:v)

Measure approximately 600 mL of water containing 0.2 % formic acid and 400 mL of acetonitrile in separate graduated measuring cylinders. Transfer the measured volumes into an appropriate vessel and mix well.

Acetonitrile / water (1:1, v:v)

Measure approximately 2000 mL of water and 2000 mL of acetonitrile in separate graduated measuring cylinders. Transfer the measured volumes into an appropriate vessel and mix well.

IV. Preparation of Standard Solutions

Stock solutions of the analytes were prepared by dissolving a weight of the test / reference item. Each stock solution was allocated a unique reference number.

The stock solutions were further diluted for use as fortification solutions in the recovery process and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and preparation of matrix-matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix which are then fortified with (intermediate) solvent standard solutions.

All solutions are stored 1 °C to 10 °C in the dark.

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

Table III: Preparation of Stock Solutions in Acetonitrile

Analyte	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
2,4-D	99.98	12.5	12.5	10	1250	S1250
2,4-DB	99.93	10.1	10.09	10	1009	S1009
MCPB	99.66	10.3	10.26	10	1026	S1026
MCPA	99.82	10.4	10.38	10	1038	S1038
Mecoprop-P	99.69	10.4	10.37	10	1037	S1037
Dichlorprop-P	99.56	10.5	10.45	10	1045	S1045
2,4-D 2EH	99.58	10.9	10.854	10.854 ¹	1000	S1000a
2,4-DB 2EH	99.67	10.9	10.864	10.864 ¹	1000	S1000a
MCPB 2EH	96.23	11.5	11.067	11.067 ¹	1000	S1000a
MCPA 2EH	99.29	11.4	11.319	11.319 ¹	1000	S1000a
Mecoprop-P 2EH	98.75	10.4	10.270	10.270 ¹	1000	S1000a
Dichlorprop-P 2EH	98.96	11.5	11.380	11.380 ¹	1000	S1000a

* taken from the Certificate of Analysis

¹ the final volume was adjusted according to the corrected weight to obtain a stock solution of exactly 1000 µg/mL. The concentration of the solution is represented in its reference number, thus this number is the same for each individual ester stock solution.

Table IV: Preparation of Fortification Solutions of Phenoxy acids in Acetonitrile / Water (1:1, v:v)

Analyte	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 MCPB MCPA Mecoprop-P Dichlorprop-P	S1250 S1009 S1026 S1038 S1037 S1045	1250 1009 1026 1038 1037 1045	0.200 0.248 0.2437 0.2408 0.2411 0.2392	25	10 each	S10c Mix
2,4-D 2,4-DB MCPB MCPA Mecoprop-P Dichlorprop-P	S10c Mix	10	2.5	25	1 each	S1b Mix

Table V: Preparation of Fortification Solutions of Esters in Acetonitrile

Analyte	Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Acid Equivalent concentration (µg/mL)	Reference of standard solution produced
2,4-D 2EH 2,4-DB 2EH MCPB 2EH MCPA 2EH Mecoprop-P 2EH Dichlorprop-P 2EH	S1000a S1000a S1000a S1000a S1000a S1000a	1000 1000 1000 1000 1000 1000	377 µL 363 µL 373 µL 390 µL 381 µL 369 µL	25	10 each	S10a Mix
2,4-D 2EH 2,4-DB 2EH MCPB 2EH MCPA 2EH Mecoprop-P 2EH Dichlorprop-P 2EH	S10a Mix	1	10	10	1 each	S1a Mix

Table VI: Preparation of Intermediate Standard Solutions of Phenoxy acids in Acetonitrile / Water (1:1, v:v)

Analyte	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 MCPB MCPA Mecoprop-P Dichlorprop-P	S1b Mix	1	1	10	0.1	S0.1c Mix

Table VII: Preparation of Solvent Calibration Solutions of Phenoxy acids in Water+0.2 % formic acid / Acetonitrile (6:4, v/v)

Reference of standard solution used	Concentration (ng/mL)	Volume taken (µL)	Final volume (µL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
S10c Mix	10	20	980	200	Std 200 ng/mL
S10c Mix	10	10	990	100	Std 100 ng/mL
S1b Mix	1	20	980	20	Std 20 ng/mL
S1b Mix	1	10	990	10	Std 10 ng/mL
S0.1c Mix	0.1	50	950	5	Std 5 ng/mL
S0.1c Mix	0.1	30	970	3	Std 3 ng/mL
S0.1c Mix	0.1	15	985	1.5	Std 1.5 ng/mL
S0.1c Mix	0.1	6	994	0.6	Std 0.6 ng/mL

Table VIII: Preparation of Matrix-matched Calibration Solutions of Phenoxy acids in Soil

Reference of standard solution used	Concentration (ng/mL)	Volume of solvent standard solution taken (µL)	Volume of extract middle solvent (Acetonitrile/ methanol) (µL)	Volume of 0.2 % formic acid in water (µL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
S10c Mix	10	20	400	580	200	mStd 200 ng/mL
S10c Mix	10	10	400	590	100	mStd 100 ng/mL
S1b Mix	1	20	400	580	20	mStd 20 ng/mL
S1b Mix	1	10	400	590	10	mStd 10 ng/mL
S0.1c Mix	0.1	50	400	550	5	mStd 5 ng/mL
S0.1c Mix	0.1	30	400	570	3	mStd 3 ng/mL
S0.1c Mix	0.1	15	400	585	1.5	mStd 1.5 ng/mL
S0.1c Mix	0.1	6	400	594	0.6	mStd 0.6 ng/mL

Matrix-matched standards of all other matrices are prepared analogously.

Table IX: Preparation of Stock Solutions for Internal Standards in Methanol / Water (1:1, v:v)

Analyte	Purity of reference item* (%)	Weighed amount of reference item (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
2,4,6-TMAA	100	10.5	10	1050	S1050
4-CDMAA	>95	12.0	10	1200	S1200

*stock solutions of internal standards were not corrected for purity

Table X: Preparation of Internal Standard Solutions in Methanol / Water (1:1, v:v)

Analyte	Reference of standard solution used	Concentration (µg/mL)	Volume taken (µL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
2,4,6-TMAA	S1050	1050	95.2	20	5 each	S5 Mix
4-CDMAA	S1200	1200	83.3			

V. Laboratory Specimen Preparation

Specimen preparation as done in this validation study is described in section 3.2 of this report.

VI. Sample Weight(s) and Fortifications

In general, control (untreated) specimens of matrices of plant origin are fortified prior to extraction with the fortification solutions as described below.

Table XI: Summary of Sample Weights and Fortifications

Fortified analyte	Matrix	Sample weight (g)	Reference of fortification solution used	Concentration of fortification solution (ng/mL)	Volume of fortification solution added (mL)	Fortification level* (mg/kg)
2,4-D 2,4-DB MCPB MCPA Mecoprop-P Dichlorprop-P	Soil	5	S1b Mix	1000	0.05	0.01
			S10c Mix	10,000	0.05	0.1
S1a Mix			1000	0.05	0.01	
S10a Mix			10,000	0.05	0.1	
2,4-D 2EH 2,4-DB 2EH MCPB 2EH MCPA 2EH Mecoprop-P 2EH Dichlorprop-P 2EH						

*for esters equivalents of the corresponding acids

VII. Sample Work-Up Procedure

1. Weigh 5 g of soil into a 40 mL glass vial with PTFE lids.
2. Fortify procedural recovery samples as required.
3. Add 10 mL of sodium hydroxide solution (47% sodium hydroxide/deionized water, 15:85, v:v) and 1 mL of methanol, ensuring that the samples are fully wetted. Cap the tubes securely and mix thoroughly by hand.
4. 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 out in a fume cupboard.
5. Allow the samples to cool until the content is luke warm, then vortex mix thoroughly.
6. Add 2.5 mL of chilled 15 N sulphuric acid to lower the pH to approx. 3. Shake vigorously by hand to mix thoroughly (release gas pressure in tube by loosening the lid slightly after initial shaking).
7. Transfer the content into a 50 mL centrifuge tube, then rinse the remaining contents of the glass vial into a the centrifuge tube with 2 mL of the chaotropic reagent 1 M monochloroacetic acid and vortex mix.
8. Add 9 mL of acetonitrile to the centrifuge tube and vortex mix.
9. To each tube add a QuEChERS Bekolut Citrat-Kit-01 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 repeat.

10. When all samples have been completed place the tube rack (horizontally) on a flatbed shaker to continue mixing for another 30 minutes.
11. Centrifuge the tube for 15 minutes at 3500 rpm at 4 °C. Pre-cool the centrifuge if necessary to reach the desired temperature.
12. Take a 1.8 mL aliquote of the middle solvent (acetonitrile/methanol) layer and transfer to a 2 mL Eppendorf tube containing the following dispersive SPE sorbents: 100 mg anhydrous magnesium sulphate, 50 mg graphitized carbon black (GCB) and 100 mg aluminium oxide.
13. Vortex mix. Continue mixing the sample on a reciprocating shaker for a further 10 minutes.
14. Micro-centrifuge the Eppendorf tubes at 13,000 rpm for 10 minutes to separate the solvent extract.
15. Take a 0.4 mL aliquot of the solvent extract and transfer to an HPLC vial and add 0.6 mL of 0.2 % formic acid in water.
16. Add 10 µL of a 5 µg/mL internal standard solution to all standards and samples.
17. Cap and mix the samples

VIII. Chromatographic and Mass Spectrometric Conditions

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

Table XII: Summary of chromatographic and mass spectrometric conditions

Chromatographic conditions				
HPLC system	1290 Infinity HPLC system, Agilent Technologies			
Pre-column	HPLC guard column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)			
Column	Phenomenex Onyx Monolithic C18, No. CH0-8158, 100 mm x 3 mm, 0 µm			
Column oven temperature	20 °C			
Injection volume	40 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]
	0.01	55	45	1000
	0.03	55	45	1000
	6.00	25	75	1000
	6.01	5	95	1000
	7.15	5	95	1000
	7.16	55	45	1000
	9.00	55	45	1000
Divert valve	1-7 min to MS			
Approx. Retention time(s)	3.3 min for 2,4-D 5.0 min for MCPB	4.5 min for MCPA 4.4 min for Mecoprop-P	4.2 min for Dichlorprop-P 4.9 min for 2,4-DB	3.6 min for 2,4,6-TMAA 4.3 min for 4-CDMAA

Summary of chromatographic and mass spectrometric conditions (continued)

Mass spectrometric conditions						
MS system	SCIEX TripleQuad 6500 System, SCIEX (Triple quadrupol mass spectrometer)					
Ionisation type	Electrospray ionization (ESI, Turbolon Spray)					
Polarity	Negative ion mode					
Scan type	Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	-4500 V	Ionspray turbo heater (TEM)			300 °C	
Curtain gas (CUR)	20 (arbitrary units)	Gas flow 1 (GS1)			30 (arbitrary units)	
Collision gas (CAD)	9 (arbitrary units)	Gas flow 2 (GS2)			40 (arbitrary units)	
Analyte monitored	Ion 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 [#]	-30	-10	-18	-7	75
	221 → 163	-35	-10	-24	-17	75
2,4-DB	247 → 161 [#]	-30	-10	-16	-17	75
	249 → 163	-38	-10	-16	-14	75
MCPB	227 → 141 [#]	-30	-10	-16	-13	75
	229 → 143	-30	-10	-16	-13	75
MCPA	199 → 141 [#]	-35	-10	-20	-15	75
	201 → 143	-15	-10	-22	-9	75
Mecoprop-P	213 → 141 [#]	-40	-10	-18	-7	75
	215 → 143	-20	-10	-18	-8	75
Dichlorprop-P	233 → 161 [#]	-40	-10	-18	-13	75
	235 → 163	-35	-10	-18	-15	75
2,4,6-TMAA	193 → 135 [#]	-40	-10	-22	-7	75
4-CDMAA	213 → 155 [#]	-25	-10	-20	-19	75

[#] used for quantification

2,4,6-TMAA was used as internal standard for the phenoxy acids 2,4-D and MCPB, for all other phenoxy acids 4-CDMAA was used as internal standard.

Instruments with similar specifications may substitute the equipment listed above. Also, instrument conditions, e. g. injection volumes, column, gradient steps, injection techniques, column oven temperature or MS parameters may be modified, but any changes must be recorded in the raw data.

Changes are acceptable, if the recoveries of fortification experiments are still in the acceptable range. Furthermore, chromatograms of control sample extracts and extracts of control samples fortified at the level of LOQ should demonstrate that a comparable level of sensitivity and selectivity is reached.

IX. Special Precautions

None

X. Calculation of Results

Quantification is performed using calibration plots with a minimum of eight (8) different concentration levels covering the required calibration range.

A calibration curve is injected prior to analysis of the samples with additional correction for the mean response of standard injections bracketing the injections of the unknown samples (typically not greater than five (5)).

The linearity of the detection system is demonstrated by use of standard solutions covering a working range which is equivalent no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level in a sample extract.

A linear regression is performed with 1/x weighting. The correlation coefficient (R) was greater or equal to 0.995.

A linear calibration function ($y = a + b \cdot x$) as determined by software Analyst, Sciex was used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_A - a}{b}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area ratio of analyte in the final solution = Peak Area Analyte / Peak Area Internal Standard (no unit) (y)
a	y -axis Intercept of the calibration curve (counts)
b	Slope of calibration curve (counts · mL/ng)

The residues are calculated by reference to the mean response of the appropriate bracketing matrix standards as follows:

$R =$	$\frac{C_1}{C_2} \times C_A \times \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte Residue (mg/kg)
C_1	Nominal concentration of bracketing matrix standards (ng/mL)
C_2	Average calculated concentration of matrix standards bracketed between samples, obtained from the matrix calibration function (ng/mL)
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)
V_{Ex}	Extraction volume (e.g. 10 mL)
V_{End}	Final volume (e.g. 1.8 mL)
V_{Ali}	Aliquot of the extract (1.8 mL)
W	Sample weight (e.g. 5 g)
DF	Dilution factor (2.5)
CF	Conversion from ng into μg (1000)

The recovery of a fortification experiment is calculated as follows:

Recovery (%) =	$\frac{R}{F} \times 100$
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

An example calculation for Soil 0.01-1 2,4-D is shown below:

$$R = \frac{c_1 * c_A * V_{Ex} * V_{End} * DF}{c_2 * W * V_{Ali} * CF}$$

$$R = \frac{3 * 1.78 * 10 * 1.8 * 2.5}{\left(\frac{2.74 + 2.53}{2}\right) * 5 * 1.8 * 1000} = 0.0101 \text{ mg/kg}$$

The following values were used in this calculation:

c_1	3 ng/mL
c_2	(2.74 ng/mL+2.53 ng/mL)/2
c_A	1.78 ng/mL
V_{Ex}	10 mL
V_{End}	1.8 mL
V_{Ali}	1.8 mL
W	5.0 g
DF	2.5

The recovery is therefore:

$$R = \frac{0.0101}{0.01} * 100 = 101\%$$

XI. Method Flow Chart